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Review Article

Bacterial battle against acidity

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Abstract

The Earth is home to environments characterized by low pH, including the gastrointestinal tract of vertebrates and large areas of acidic soil. Most bacteria are neutralophiles, but can survive fluctuations in pH. Herein, we review how *Escherichia, Salmonella, Helicobacter, Brucella*, and other acid-resistant Gram-negative bacteria adapt to acidic environments. We discuss the constitutive and inducible defense mechanisms that promote survival, including proton-consuming or ammonia-producing processes, cellular remodeling affecting membranes and chaperones, and chemotaxis. We provide insights into how Gram-negative bacteria sense environmental acidity using membrane-integrated and cytosolic pH sensors. Finally, we address in more detail the powerful proton-consuming decarboxylase systems by examining the phylogeny of their regulatory components and their collective functionality in a population.

Keywords: Gram-negative bacteria, protons, glutamate acid-dependent acid resistance, lysine-dependent acid resistance, pH-sensor

Introduction

Compared to acidophilic organisms, which grow optimally at a pH near 3.0, and alkaliphiles, which prefer a pH between 8 and 10.5, most bacteria are neutralophilic and grow best at pH values between 7.0 and 7.8 (Slonczewski et al. 2009). However, neutralophilic bacteria are able to adapt and grow in environments with pH values ranging from 5.5 to 9.0, and thereby occupy a wide range of environmental niches including acidic environments such as human and animal digestive systems, acidic soils, and fermented foods (Fig. 1), which has important effects on the environment and public health. The evolution of molecular systems conferring acid tolerance and/or acid resistance (AR) is an important survival strategy for these bacteria, including the pathogenic ones (Castanié-Cornet et al. 1999).

The pH of the human stomach ranges between 1 and 3 under fasting conditions and increases during digestion. The stomach, thus is a major bactericidal barrier against enterobacteria (Martinsen et al. 2005, Kalantzi et al. 2006). Its low pH causes growth arrest and even death of bacteria (e.g. only 10% of the highly resistant *E. coli* population survives in the stomach). Under this condition, an acidification of the bacterial cytoplasm occurs as protons (H^+) use water bridges or weak organic acids to traverse the cytoplasmic membranes (Brahmachari et al. 2019). This acidification has two important effects: slowing biological activity of macromolecules and decreasing or abolishing the pH difference at the cytoplasmic membrane. The latter is an important component of the proton motive force and crucial for bioenergetics (Foster 2004, Lund et al. 2014).

Neutralophilic enterobacteria that survive the stomach also confront mild acid stress (pH 6.4 or lower) in the colon due to the presence of short-chain fatty acids (Hirshfield et al. 2003), such as butyric, propionic, lactic, acetic, and formic acids, which are produced by anaerobic fermenting bacteria (Evans et al. 1988, Boets et al. 2015). Intracellular pathogens, such as *Salmonella*, also encounter low-pH (pH 4–5) environments in the macrophage phagosome (Rathman et al. 1996, Steinberg et al. 2007) (Fig. 1).

On Earth there are large areas of soil with an acidic pH-value (pH 6.5–3.5; Wieder et al. 2014; Fig. 1). Many processes contribute to soil acidification. For example, carbon dioxide (CO_2) emissions combine with water in the atmosphere to form carbonic acid (H_2CO_3), and the burning of fossil fuels releases oxides of sulfur and nitrogen into the atmosphere that react with water to form sulfuric and nitric acids. This acidic rain then accumulates in the soil. Root respiration and decomposition of organic matter by microorganisms also release CO_2, which increases the level of H_2CO_3 in the soil. Simultaneously, cations are exchanged resulting in leaching of base cations, such as Ca^{2+}, Mg^{2+}, from the soil and an increase of the percentage of Al^{3+} and H^+ (vanLoon 1984). Severely acidic conditions also can form in soils located near acid mine drainage sites (Johnson and Hallberg 2005).

In food industry, acidity plays an important role for bacteria (Fig. 1). Homofermentative and heterofermentative lactic acid bacteria and propionic bacteria, which are predominant in dairy products, trigger the production of lactic acid, propionic acid, ethanol, and CO_2, all of which enhance flavor, digestibility, nutritional properties, and preservation (Fröhlich-Wyder et al. 2017, Siedler et al. 2019). As a consequence, these bacteria are exposed to acidity of their own fermentation activities. On the other hand, weak acid preservatives such as potassium sorbate, calcium propionate, and sodium benzoate are often added to foods to inhibit the growth of bacteria and to extend the shelf life of products (Guynot et al. 2005).

In this review, we focus on the manifold adaptive responses of neutralophilic Gram-negative proteobacteria and the molecular mechanisms of sensing acid stress. For detailed information on

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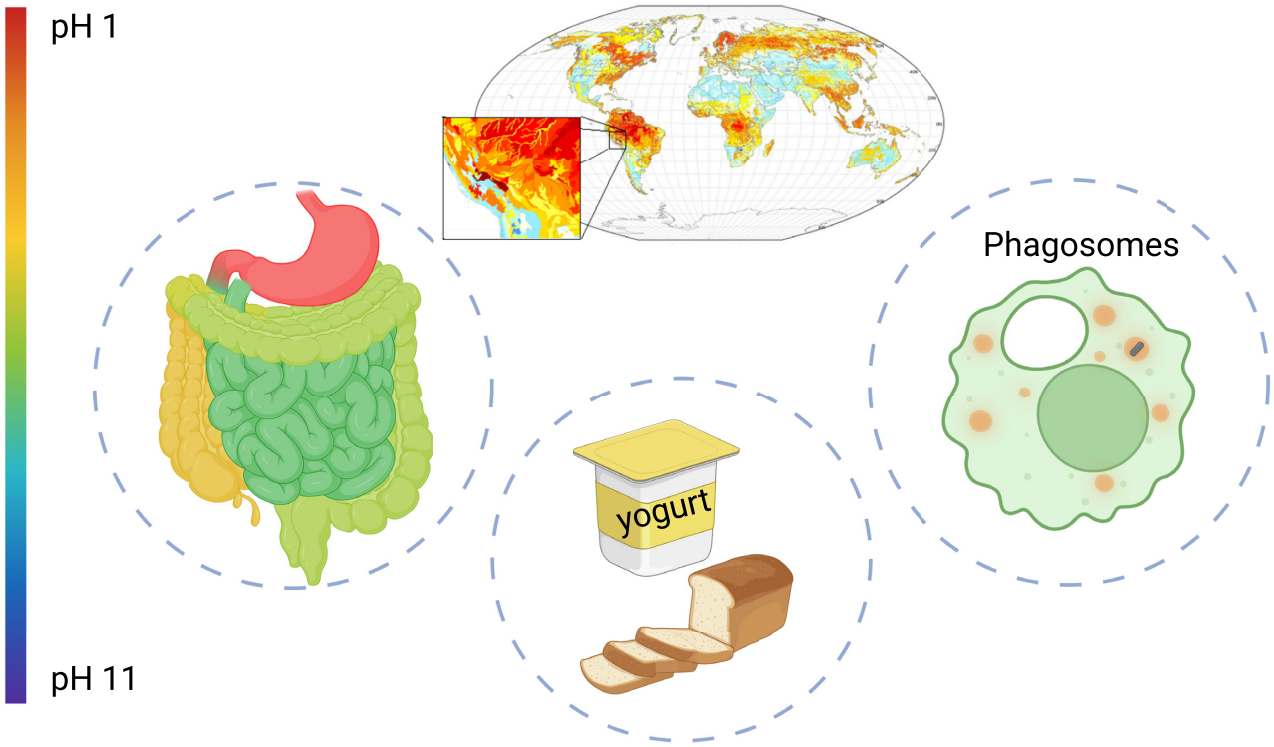

Figure 1. Schematic overview of acidic environments encountered by bacteria.

the acid-stress response of neutralophilic Gram-positive bacteria, see reviews by Guan and Li (2020) and Arcadi et al. (2020).

Acid stress adaptation of Gram-negative γ-proteobacteria

pH homeostasis and ion transport

In response to rapid drops in external pH, prokaryotic cells can retain a fairly constant internal pH from 7.5 to 8.0 for neutralophiles, 6.5 to 7.0 for acidophiles, and 8.4 to 9.0 for alkaliphiles (Booth 1985). In contrast to the periplasm, the cytoplasm transiently acidifies and restores neutral pH within short time intervals due to numerous cellular acid stress adaptions (Wilks and Slonczewski 2007, Krulwich et al. 2011). An important factor for preserving a constant internal pH is the intrinsic buffering capacity of the cytoplasm (Fig. 2). Excess intracellular protons can be sequestered by pH-titratable cellular components, such as amino acid sidechains, polyamines, polyphosphates, or inorganic phosphates, as summarized in Slonczewski et al. (2009).

The cytoplasmic membrane constitutes a major barrier to protons due to the intrinsic impermeability of the lipid bilayer and specifically controlled ion influx (Booth 1985). However, protons can pass this natural barrier using transient water chains, channels, or damaged membranes (Deamer 1987, Lund et al. 2014). Different types of acid stress counteracting membrane-integrated transporters exist, such as monovalent cation-proton antiporters, or proton pumps associated to the respiratory chain (Fig. 2).

The active transport of H^+ is a constitutive mechanism that maintains a constant internal pH, but the activity of H^+ pumps is constrained by the generation of the proton motive force (Mitchell 1973). In low-pH conditions, *E. coli* downregulates synthesis of the F_oF_1-ATP synthase to limit proton re-entry (Maurer et al. 2005, Sun et al. 2012). In various other organisms, the F_oF_1-ATP synthase relieves acid stress via ATP-dependent extrusion of protons from the cytoplasm (Kobayashi et al. 1986, Foster and Hall 1991, Cotter et al. 2000, Sun et al. 2012). Net potassium uptake is the dominant means of maintaining ion homeostasis (Booth 1985).

Acidophilic bacteria also can relieve acid stress by reversing the usually negative transmembrane potential ($\Delta\psi$) Matin 1999). An inside-positive membrane potential helps prevent further proton uptake due to charge repulsion (Matin et al. 1982). A similar mechanism has been suggested for neutralophilic bacteria (Foster 2004, Richard and Foster 2004). In addition, in *E. coli*, two genes (*eriC* and *mriT*) coding for two chloride channels are induced at extremely low pH levels. Export of Cl^- prevents H^+ uptake and restores an inside-negative $\Delta\psi$ once the external pH is recovered (Iyer et al. 2002, Accardi and Miller 2004).

Enzyme-based H^+-consuming acid stress resistance systems

A total of four different H^+-consuming AR systems have been identified in *E. coli* (Figs. 2 and 3). These are the glutamate–dependent AR2 (Gad) system (also known as GDAR), the arginine-dependent AR3 (Adi) system (also known as ADAR), the lysine-dependent AR4 (Cad) system (also known as LDAR) and the ornithine-dependent AR5 (Orn) system (also known as ODAR). Each of these four systems relies on a cytoplasmic amino acid decarboxylase and a corresponding antiporter. In an H^+-consuming reaction, the pyridoxal-5'-phosphate (PLP)-dependent decarboxylase converts a specific amino acid (e.g. glutamate, arginine, lysine, and ornithine) into a more alkaline product, which is then exported by a specific antiporter in exchange for the amino acid. Through these biochemical reactions, the internal and external pH levels rise. The AR2–AR5 systems are activated at different pH ranges (< 6) Fig. 3) with varying strengths in individual cells, and each decarboxylase has its own optimum pH (Gale 1940, 1946, Lin

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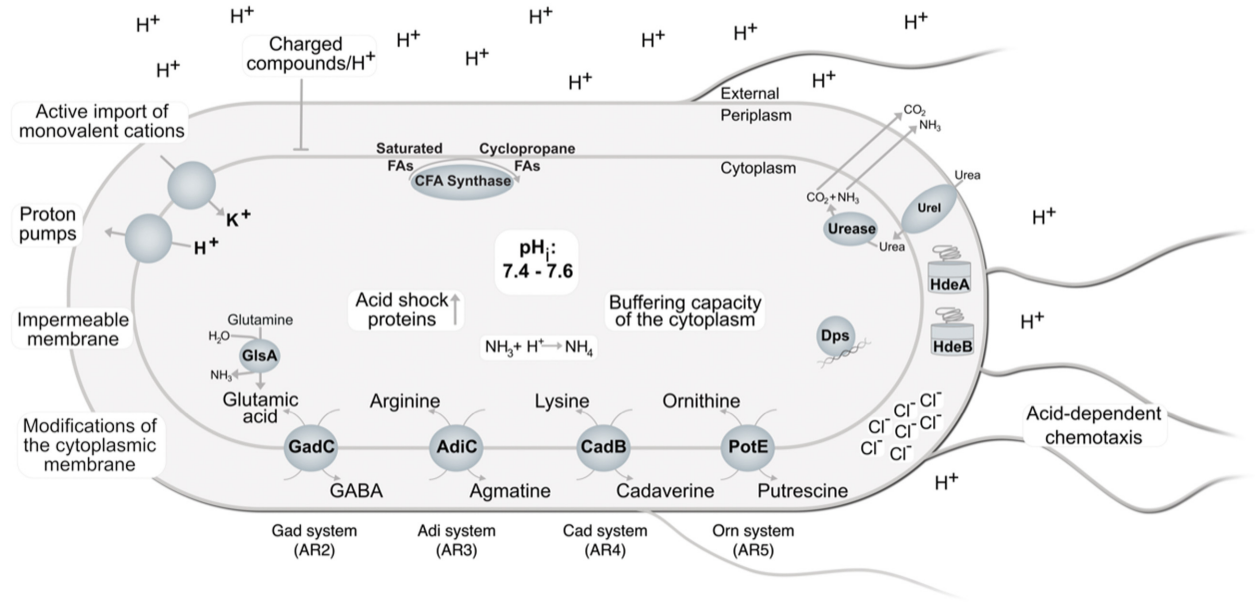

Figure 2. Overview of mechanisms in Gram-negative proteobacteria to cope with acidic environments. The various mechanisms to counteract acidic stress are discussed in detail in the text.

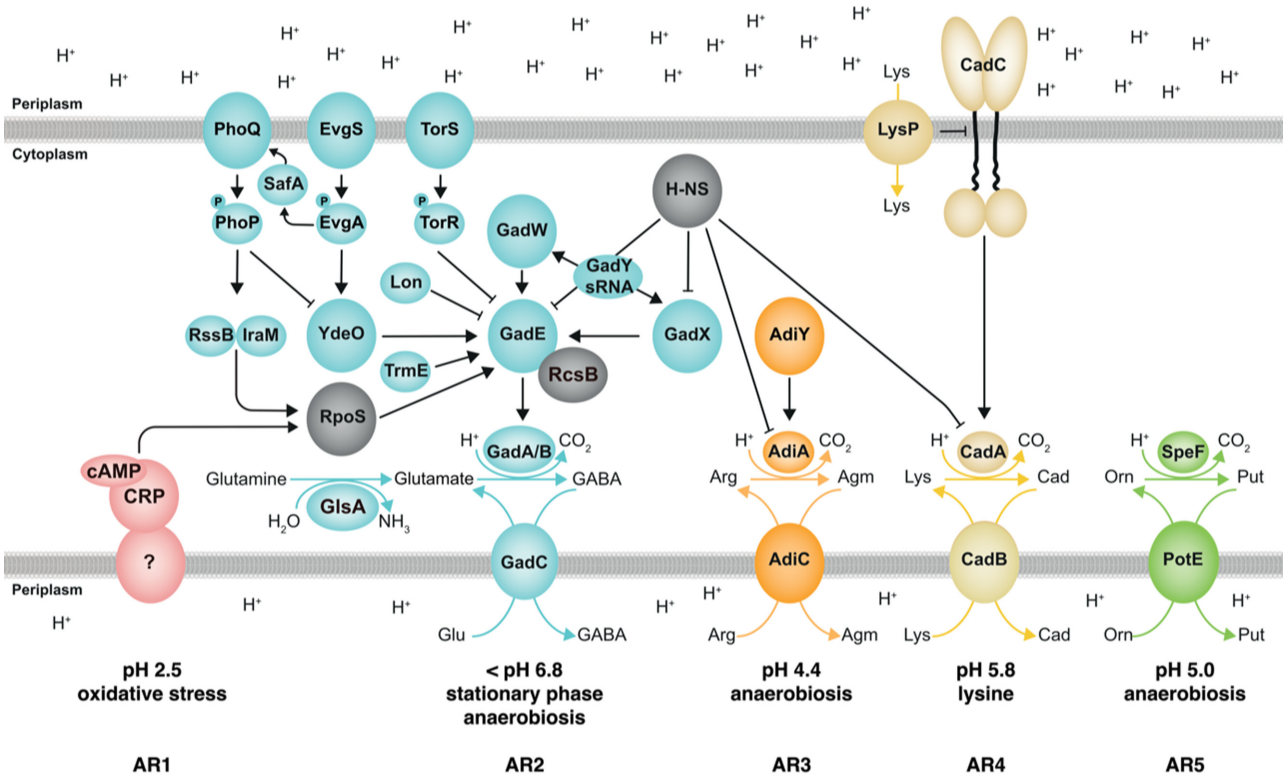

Figure 3. The regulatory network of the enzyme-based acid stress resistance systems in *E. coli*. Regulated induction of AR1 (in red), the Gad (AR2; in light blue), Adi (AR3; in orange), Cad (AR4; in pale yellow), and Orn (AR5; in green) systems requires a network of membrane-integrated pH-sensors, namely EvgS, PhoQ, and CadC; interconnected transcription factors; and the sRNA GadY. The glutamate decarboxylases GadA and GadB convert glutamate (Glu) into γ-aminobutyrate (GABA), which is excreted by GadC. The glutaminase GlsA (formerly YbaS) converts glutamine in glutamate and NH_3. The arginine decarboxylase AdiA converts arginine (Arg) into agmatine (Agm), which is excreted by AdiC. The lysine decarboxylase CadA converts lysine (Lys) into cadaverine (Cad), which is excreted by CadB. The ornithine decarboxylase SpeF converts ornithine (Orn) into putrescine (Put), which is excreted by PotE. RpoS, RcsB, and H-NS (gray symbols) are involved in the regulation of several enzyme-based acid stress resistance systems. Adapted from Lin et al. (1996), Castanié-Cornet et al. (1999), Foster (2004), Richard and Foster (2004), Bougdour et al. (2008), Zhao and Houry (2010), Eguchi et al. (2011), and Lund et al. (2014). The stimuli leading to an induction of each of the five AR systems are indicated below each system.

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et al. 1995, Bearson et al. 1997, Castanié-Cornet et al. 1999, Foster 2004, Kanjee et al. 2011b, Kanjee and Houry 2013, Aquino et al. 2017). Little is known about AR1, an oxidative or glucose repressed system, which is independent of an external amino acid.

Acid-resistance system 1 (AR1)

The AR1 system is induced in stationary phase cells in complex media, or in minimal medium under extremely acidic stress conditions (pH 2.5) (Lin et al. 1996, Castanié-Cornet et al. 1999, Foster 2004). Its expression is regulated by alternative sigma factor σ^S (RpoS) and the cyclic adenosine monophosphate (cAMP) receptor protein (CRP). Part of the protective function of the AR1 system is the F_oF_1-ATP-synthase, which hydrolyzes ATP to pump protons out of the cell and lower internal pH (Richard and Foster 2004). Aquino et al. (2017) predicted a model according to which the AR1 system uses the enzymes of the Gad system, which are described in the next chapter, to decarboxylate intracellular glutamate.

Glutamate-dependent AR (Gad) system (AR2)

The core components of the Gad system are the decarboxylase isoenzymes GadA and GadB and the glutamate/γ-amino butyrate antiporter GadC in *E. coli*. Both GadA and GadB convert L-glutamate into γ-amino butyrate (GABA), consuming a proton in this process. The *gadBC* genes form one operon, while *gadA* is located inside the acid fitness island in *E. coli* (Biase and Pennacchietti 2012). The Gad system is induced during transition to the stationary phase and during exponential growth in acidified media (Biase et al. 1999, Castanié-Cornet et al. 1999, Castanié-Cornet and Foster 2001, Weber et al. 2005). In addition, the Gad system is essential for survival at pH levels below 3, but is also induced during anaerobiosis and salt stress (Biase et al. 1999, Hayes et al. 2006).

GadA and GadB form hexamers, have maximal activities at pH 3.7–3.8 (Shukuya and Schwert 1960, Lawson and Quinn 1967) and are 98% identical at the nucleotide level (Smith et al. 1992). More functional and structural details of GadB are summarized in excellent reviews of Biase and Pennachietti (2012) and Zhao and Houry (2010).

The high-resolution X-ray crystal structure of GadC, determined by Ma et al. (2012), reveals detailed insights into substrate binding and pH-dependent transport activity. GadC antiport activity has been observed at pH $\leq$ 6 *in vitro*, and *in vivo* measurements of GABA export suggest maximum activity at pH $\leq$ 3 (Richard and Foster 2004, Ma et al. 2012). Notably, GadC recognizes substrates by their net electric charge and exclusively transports $Glu^{0/-1}$ or Gln^0 in exchange for $GABA^{+1}$ (Ma et al. 2013, Tsai et al. 2013). Under nonactivating conditions, a C-terminal plug in GadC prevents substrate-binding. Correspondingly, mutants lacking the C-plug display a shift in pH-dependent transport activity towards higher pH-units (Ma et al. 2012).

In addition to glutamate, GadC also transports the amino acid glutamine (Ma et al. 2012, 2013), which is subsequently converted to glutamate by the glutaminase GlsA (formerly YbaS; Figs. 2 and 3). During this reaction, NH_3 is released, which itself sequesters protons while the produced glutamate serves as further substrate for GadA and GadB (Lu et al. 2013). Correspondingly, *glsA* is upregulated under the same conditions as *gadBC* (Biase and Pennacchietti 2012). In contrast to substrates and reaction products from other AR systems, glutamate and GABA both contribute to cytoplasmatic buffering due to carboxyl groups with suitable pK_a values (Richard and Foster 2004, Lund et al. 2014).

The transcriptional regulation of *gadA*, *gadB*, and *gadC* represents one of the most complex examples thereof within bacteria. More than 20 regulatory elements with partially interconnected circuits and several small noncoding RNAs are involved in controlling their expression (Foster 2004, Zhao and Houry 2010).

The central activator of the Gad system is the transcriptional regulator GadE, which belongs to the LuxR-family (Ma et al. 2003; Fig. 3). GadE binds to the so-called *gad* box, a 20-nt region 63 bp upstream of the *gadA* and *gadBC* transcriptional start site (Castanié-Cornet and Foster 2001, Ma et al. 2002). In stationary phase *E. coli* cells, the 3´end of the GadE mRNA is processed and a 91-nt small regulatory RNA, GadF, is generated, which is involved in regulation of acid resistant genes (Melamed et al. 2016). To initiate expression of *gadA*, GadE forms a heterodimer with RcsB (Castanié-Cornet et al. 2007, 2010). RcsB is the response regulator (RR) of the Rcs phosphorelay system, which is responsible for adapting to cell envelope stress in *Enterobacteriaceae* (Meng et al. 2021). Beyond RcsB, the Rcs system consists of the transmembrane hybrid histidine kinase (HK) RcsC, another transmembrane protein RcsD and the outer membrane protein RcsF. RcsC and RcsD dephosphorylate RcsB in the absence of envelope stress. RcsB is not only able to form heterodimers with GadE, but also with RcsA, MatA, DctR, BglJ, and presumably EvgA, to control numerous target genes (Castanié-Cornet et al. 2010, Venkatesh et al. 2010, Johnson et al. 2011, Pannen et al. 2015).

gadE transcription is regulated by the AraC/XylS-like regulator YdeO of the EvgS/EvgA-PhoQ/PhoP-YdeO circuit (Fig. 3). YdeO and EvgA have additive effects on *gadE* transcription (Ma et al. 2004). EvgA also contributes indirectly to *gadE* transcription, on the one hand through activation of YdeO by phosphorylation and on the other hand by induction of the PhoQ/PhoP HK/RR system via the small connector protein SafA (Ma et al. 2004, Eguchi et al. 2012, Ishii et al. 2013). The PhoQ/PhoP system in turn activates the anti-adaptor protein IraM, which inhibits activity of the protease adaptor RssB. RssB is responsible for degradation of RpoS by ClpXP, which implies that an active PhoP leads to increased RpoS levels (Eguchi et al. 2011).

The GadX-GadY-GadW regulatory circuit is required for induction of the Gad system in stationary phase cells, independently of the culture conditions (Ma et al. 2002), as shown in Fig. 3. GadW and GadX both belong to the AraC/XylS family and activate *gadA* and *gadBC* directly and indirectly through transcriptional activation of *gadE* (Ma et al. 2002, Tramonti et al. 2006, Sayed et al. 2007). Both regulators exert a dual role and can also repress the above-mentioned genes when bound to the *gad* box (Tramonti et al. 2002). Another element adding even more complexity to this circuit is the small RNA GadY. GadY directly base pairs and thereby stabilizes the 3´ untranslated region of the *gadX* mRNA (Opdyke et al. 2004). Another factor linked to AR via GadW is the LuxR-like protein SdiA, which presumably regulates *gadW* and *gadY* at the transcriptional level (Ma et al. 2020). This effect is further influenced by N-acyl homoserine lactones, which stabilize the SdiA protein and increase its binding affinity to DNA (Nguyen et al. 2015, Ma et al. 2020).

Other elements, including CRP and several sRNAs, influence induction of the Gad system through RpoS. Increased amounts of RpoS promotes *gadE*, *gadX*, and *gadY* expression (Castanié-Cornet and Foster 2001, Ma et al. 2002). For example, cAMP-CRP negatively controls *rpoS* transcription during exponential growth (Lange and Hengge-Aronis 1994). The DsrA small noncoding RNA stabilizes the *rpoS* mRNA and simultaneously inhibits *hns* mRNA translation via RNA–RNA interactions, which is expected to reduce gene silencing caused by the histone-like nucleoid-structuring protein

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(H-NS) (Lease et al. 2004). This contributes to AR as H-NS represses *gadA*, *gadE*, and *gadX* (Giangrossi et al. 2005). The expression of *rpoS* is also positively regulated by the GcvB, RprA, and ArcZ small RNAs (Jin et al. 2009, Bak et al. 2014).

The expression of *gadE* is also induced during anaerobic growth of *E. coli* via the MnmE (TrmE) circuit. The Era-like GTPase MnmE can indirectly activate *gadE* in stationary-phase cells in rich medium supplemented with glucose (Gong et al. 2004). This activation also appears to be associated with anaerobic conditions and the ArcA/ArcB HK/RR system (Deng et al. 2013).

A combined ChIP-Seq RNA-Seq approach by Aquino et al. (2017) reports binding of the carbon starvation–induced regulator CsiR to *gadX*, *ydeO*, and *gadW* promoter regions, as well as effects of CsiR on gene expression of multiple *gad* genes. In addition to vast amounts of direct and indirect activators, examples of negative regulation of the Gad system also have been reported. In addition to H-NS, the RR TorR also negatively regulates *gad* genes, and the Lon protease is involved in GadE proteolysis (Bordi et al. 2003, Heuveling et al. 2008).

In summary, the Gad system is the most important AR system under extremely acidic conditions. In *E. coli* and other bacteria (see below), this system includes several decarboxylase isoenzymes. Up to now, no differences have been demonstrated in the induction of *gadA* and *gadB* and protein function.

It would be interesting to find out why two nearly identical decarboxylase genes have evolved or to find a condition in which only one of the two enzymes is produced. It also remains puzzling why the network of transcriptional regulators and small RNAs controlling the Gad system reaches such enormous complexity in *E. coli*. In contrast, the molecular design of the Cad, Adi, and Orn systems is simpler.

Arginine-dependent AR (Adi) system (AR3)

The arginine-dependent AR (Adi) system, shown in Fig. 3, consists of the arginine decarboxylase AdiA, the arginine/agmatine transporter AdiC, and the AraC/XylS-like regulator AdiY. The *adiA*, *adiY*, and *adiC* genes are clustered but not arranged in an operon, as *adiY* separates *adiA* and *adiC* on the chromosome. This system is maximally activated in *E. coli* grown in rich medium with an external pH of 4.4 under anaerobic conditions (Melnykovych and Snell 1958, Blethen et al. 1968, Kanjee and Houry 2013). AdiA decarboxylates arginine to produce agmatine, which is then secreted by AdiC via an exchange with extracellular arginine.

AdiA activity is pH-dependent and regulated via changes in oligomerization. At pH 5.2, AdiA mainly forms decamers. Oligomers dissociate into inactive dimers at higher pH values, and this transition is enhanced through electrostatic repulsion because AdiA has many negatively charged surface residues (Nowak and Boeker 1981).

Like AdiA, the activity of AdiC is pH-dependent, increasing at extremely acidic pH levels (< ~4) compared to physiological pH (Fang et al. 2007). The outward- and inside-facing domains of the antiporter also are sensitive to pH. AdiC functions as a homodimer, but each monomer can exchange one arginine to one agmatine (Fang et al. 2007, Gao et al. 2009, 2010, Kowalczyk et al. 2011, Krammer et al. 2016, 2018, Ilgü et al. 2018). The maximum uptake for arginine from the periplasm is reached at pH < 4, and the optimal pH for the cytoplasm is 5.5, showing that AdiC is a pH sensor of the periplasm and the cytoplasm (Gao et al. 2009, Zomot and Bahar 2011, Tsai and Miller 2013, Wang et al. 2014, Krammer et al. 2018).

Transcription of the *adi* genes is influenced by the growth phase, the switch to anaerobic conditions and several transcriptional regulators. Nevertheless, the main transcriptional regulator of the Adi system is AdiY, a member of the AraC/XylS-family, which activates the transcription of its target genes *adiA* and *adiC* (Stim-Herndon et al. 1996). *adiY* transcription itself is downregulated upon entry into the stationary phase of *E. coli* (Smith et al. 2018) and *adiY* mRNA can be base-paired by the small RNA SgrS, resulting in post-transcriptional downregulation under anaerobic conditions (Bobrovskyy and Vanderpool 2015). In addition to the main activator AdiY, CysB, CsiR, and H-NS have modulating effects on the expression of the Adi system (Shi and Bennett 1994, Aquino et al. 2017).

Recently, it was shown that pH-dependent activation of the *adiA* promoter can be manipulated by changes in intracellular pH, as activation of the Adi system can be influenced by manipulation of the cytosolic proton concentration either by deletion of *cadA* or by overproduction of *cadA* (triggered by increased CadC copy numbers) (Brameyer et al. 2022). These data suggest that AdiY acts as an intracellular pH sensor that detects a decrease in cytoplasmic pH (Brameyer et al. 2022). The molecular mechanism of how AdiY responds to low pH is still unclear. But it is an interesting hypothesis that low cytosolic pH, which normally has deleterious effects, is sensed by one sensor to activate another AR system.

Lysine-dependent AR (Cad) system (AR4)

The Cad system consists of the lysine decarboxylase CadA, the lysine/cadaverine antiporter CadB and the regulatory protein CadC, a member of the ToxR-family (Fig. 3). This system only moderately protects the cells from extreme acid shock, but it is important for growth in mildly acidic environments (Watson et al. 1992, Dell et al. 1994). Together with the Gad system, the Cad system contributes to cell survival in anaerobic environments with fermentation acids and phosphate starvation, such as the small intestine (Moreau 2007).

CadC functions as a periplasmic pH sensor and regulates the expression of *cadA* and *cadB*. Like all members of the ToxR-family, CadC is a membrane–integrated sensor with DNA-binding capacity. The *cadA* and *cadB* genes are organized in one operon, which is activated by CadC in *E. coli* cells exposed in mildly acidic conditions (pH 5.8) and in the presence of lysine (> 1 mM) (Watson et al. 1992, Neely et al. 1994, Neely and Olson 1996, Soksawatmaekhin et al. 2004, Tetsch et al. 2008, Fritz et al. 2009). Detection of low pH leads to dimerization of the periplasmic sensory domain of CadC. A disordered CadC linker on the cytoplasmic side is required to transduce the pH-dependent response into a structural rearrangement that facilitates dimerization of the cytoplasmic DNA-binding domain (Haneburger et al. 2011, Buchner et al. 2015, Schlundt et al. 2017). By recruiting the RNA polymerase, CadC binds to the *cadBA* promoter at two specific binding sites, with each binding site occupied by a CadC homodimer (Neely et al. 1994, Kuper and Jung 2005, Eichinger et al. 2011, Haneburger et al. 2011, Lindner and White 2014, Schlundt et al. 2017).

The availability of external lysine is transduced to CadC by the cosensor and inhibitor LysP, a lysine-specific permease in *E. coli*. CadC is inactivated by an interaction with LysP in low-lysine conditions. This interaction is released when lysine is abundant (Tetsch et al. 2008, Rauschmeier et al. 2014). Additionally, CadC activity is negatively feedback-inhibited by cadaverine (Fritz et al. 2009, Haneburger et al. 2011, 2012). LysP and H-NS act as negative regulators of the Cad system in *E. coli*. H-NS binds under noninducing conditions to four potential binding sites within the *cadBA*

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promoter region and forms a repressor complex. This complex dissolves when CadC binds to the Cad1 site. Binding of CadC to Cad2 initiates transcription of *cadBA* (Shi et al. 1993, Neely et al. 1994, Kuper and Jung 2005, Buchner et al. 2015, Schlundt et al. 2017).

In *E. coli*, *cadC* transcription is constitutive (Watson et al. 1992, Dell et al. 1994), but *cadC* translation depends on the elongation factor P (EF-P) due to the presence of a polyproline motif. EF-P is an elongation factor that enhances translation of polyproline-containing proteins through alleviating of the ribosome stalling. This translational regulation keeps the copy number of CadC low at only 1–3 copies per cell, on average (Ude et al. 2013). Brameyer et al. (2019) showed that a fluorophore-tagged CadC appears uniformly in the membrane of cells exposed to physiological pH. Under CadC-activating conditions, a distinct cluster appears at the lateral cellular position, which immediately disappears under nonstress conditions. The results suggest that binding of CadC to DNA determines its localization in the cell. This finding is confirmed using various CadC variants that mimic the active state of CadC, independent of environmental stimuli, or in mutants in which the DNA-binding site is relocated (Brameyer et al. 2019, Martini et al. 2021). Due to the low copy number and consequent stochastic distribution of CadC in single *E. coli* cells, the Cad system is heterogeneously activated, resulting in about two thirds of cells in an ON state and the rest in an OFF state (Brameyer et al. 2020). For more details on the molecular mechanism of pH-sensing by CadC, see below.

CadA catalyzes the decarboxylation of L-lysine into the more alkaline polyamine cadaverine and into CO_2 by consuming a proton. The enzymatic activity is regulated by oligomerization and the alarmone guanosine 3′,5′-bis(diphosphate) (ppGpp). Active CadA comprises five dimers that form a decamer in a double-ringed structure with a distinct 5-fold symmetry homologous to AdiA (Snider et al. 2006). A total of 10 molecules of the stringent response molecule ppGpp can bind to a CadA decamer to inhibit activity (Kanjee et al. 2011a), enabling cells to react quickly to changing environmental conditions. The maximum activity for CadA occurs at pH 5.7 (Gale and Epps 1944, Sabo et al. 1974, Kanjee and Houry 2013).

The structure of the lysine/cadaverine antiporter CadB is presently unknown. CadB performs an electrogenic exchange of lysine with cadaverine. At physiological pH, CadB mediates uptake of cadaverine, i.e. driven by proton-motive force (Soksawatmaekhin et al. 2004, 2006).

The Cad system is also important for survival and growth of other pathogenic bacteria in acidic conditions, such as S. Typhimurium (Park et al. 1996) and *V. cholerae* (Merrell and Camilli 1999). However, regulation of the Cad system varies among species. For example, in *Vibrio campbellii*, the Cad system is the major pH stress response system, but its Cad module is missing the cosensor LysP. In *Vibrio* spp., *cadC* expression is activated by the LysR-type transcriptional activator AphB (Rhee et al. 2006, Kovacikova et al. 2010). Another difference compared to *E. coli* is that the translation of CadC does not depend on EF-P. Consequently, CadC has higher copy numbers of about 41–69 copies per cell under activating conditions. The Cad system, thus is homogeneously activated in all cells of the *V. campbellii* population (Brameyer et al. 2020).

Ornithine-dependent AR (Orn) system (AR5)

The Orn system is an exceptional AR system because it converts a nonproteinogenic amino acid. The system consists of the inducible ornithine decarboxylase SpeF and the ornithine/putrescine antiporter PotE (Fig. 3). The main activity of this system occurs during anaerobic growth of *E. coli* at low pH and high ornithine levels (Gale 1946, Applebaum et al. 1977, Kashiwagi et al. 1991, 1992). The genes *speF* and *potE* are arranged in one operon and induced at pH ∼5.0. Dimerization and GTP regulate the decarboxylase activity, and the alarmone ppGpp binds weakly to SpeF (Applebaum et al. 1977, Kanjee et al. 2011b). The optimal enzymatic activity for SpeF occurs at pH 7.0. This relatively high optimal pH, in comparison to other amino acid decarboxylases, highlights the efficiency of these four systems to deal with extremely acidic conditions (Gad > Adi > Cad >> Orn) (Foster 2004, Kanjee and Houry 2013). Under mildly acidic conditions, PotE exchanges ornithine or lysine with putrescine. At neutral pH, PotE catalyzes the proton motive force–dependent uptake of putrescine (Kashiwagi et al. 1992, 1997). Regulation of the *speF-potB* operon is unusual and involves ribosome stalling and antitermination (Ben-Zvi et al. 2019, Herrero Del Valle et al. 2020). Upstream of *speF* is a short open reading frame (ORF) that encodes the 34-amino acid peptide SpeFL (also known as *orf34*). This ORF is conserved in many γ-proteobacteria. In the absence of ornithine, translation of *speFL* causes ribosome stalling and transcription of *speF* pauses. In the presence of ornithine, translation of SpeFL activates *speF* expression by preventing Rho-dependent transcription termination (Herrero Del Valle et al. 2020).

Enzyme-based ammonia-producing AR systems

Deiminase and deaminase systems

Different bacterial species, such as *Helicobacter pylori*, *Pseudomonas aeruginosa*, and many Gram-positive bacteria like *Streptococcus mutans*, use deiminase and deaminase systems to withstand acidic environments. These enzymes produce ammonia (NH_3) to neutralize internal protons, which are used to create ammonium ions (NH_4^+) and in turn increase the internal pH. Different deiminase and deaminase systems are present in different groups of bacteria in varying combinations.

The agmatine deiminase (AgD) belongs to the family of guanidino-group modifying enzymes (GMEs) (Linsky and Fast 2010). It catalyzes the conversion of agmatine to N-carbamoyl putrescine and NH_3. AgD mediates acid resistance in *Streptococcus mutans*, *P. aeruginosa* and *H. pylori* (Jones et al. 2010).

Another GME is the L-arginine deiminase, it belongs to the arginine deiminase system (Linsky and Fast 2010), which protects cells against acid damage (Cunin et al. 1986, Marquis et al. 1987, Liu et al. 1995, D'Hooghe et al. 1997, Angelis et al. 2002). This system is present in a variety of bacteria, including *Mycoplasma*, *Pseudomonas, Bacillus*, *Rhizobium*, and lactic acid bacteria. A total of three enzymes are responsible for its acid tolerance: (i) arginine deiminase, which degrades arginine into citrulline and NH_3, (ii) ornithine carbamoyl transferase (OTC), which cleaves citrulline into carbamoyl phosphate and ornithine, and (iii) carbamate kinase, which produces ATP, NH_3, and CO_2 through dephosphorylation of carbamoyl phosphate. These enzymes show maximal activity at a pH of 3.1 or lower (Cunin et al. 1986, Marquis et al. 1987, Casiano-Colón and Marquis 1988). The generation of NH_3 and of one ATP per arginine contributes to the internal pH homoeostasis through the F_oF_1-ATPase, which extrudes cytoplasmic protons.

In *E. coli*, the glutaminase GlsA (formerly YbaS) is part of the Gad system, i.e. activated under acidic conditions (Figs 2 and 3). L-glutamine is imported by GadC and then converted by GlsA to

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L-glutamate and NH_3 (Ma et al. 2012, 2013, Sun et al. 2012, Lu et al. 2013). Another deaminase system in *E. coli* is based on adenosine. Internal pH can increase when adenosine is converted into inosine and NH_3, a reaction catalyzed by the adenosine deaminase Add (Maurer et al. 2005, Sun et al. 2012).

Urease

Another enzyme frequently used to combat acidification is urease, which is present in a wide variety of bacterial genera (e.g. *Helicobacter*, *Brucella*, *Klebsiella*, *Pseudomonas*, *Yersinia*, *Proteus*, *Staphylococcus*, and *Streptococcus*) (Lund et al. 2014), as well as members of the human gut microbiota (Mora and Arioli 2014). Urease catalyzes the hydrolysis of its substrate urea to NH_3 and carbamate, which subsequently decomposes into another molecule of NH_3 and CO_2 (Mobley et al. 1995). NH_3 contributes to cytoplasmic and periplasmic buffering by neutralizing protons and yielding NH_4^+ (Scott et al. 2010), and CO_2 is converted to bicarbonate by alpha-carbonic anhydrase (Marcus et al. 2005). The *ureAB* genes encode the urease subunits, whereas the protein products of *ureDEFG* are involved in urea metabolism, urease biogenesis and incorporation of nickel (Lund et al. 2014). Nickel ions are essential cofactors of urease activity and are inserted into the active site of the enzyme. Additionally, the NikR transcription factor of *H. pylori* regulates nickel uptake and induces expression from the *ureA* promoter in low-pH conditions (van Vliet et al. 2002, Li and Zamble 2009).

The importance of urease activity is best studied in *H. pylori*, in which the proton-gated urea channel UreI mediates urea uptake into the cytoplasm. Urea is present in 1–3 mM concentrations in gastric juice. UreI activity is strictly pH dependent, which prevents urea import and alkalization of the cytoplasm during physiological pH conditions (Weeks et al. 2000). Correspondingly, the previously mentioned genes encoding urease subunits are regulated in a pH-responsive manner, mediated by the ArsS/ArsR HK/RR system (see below) and the cytoplasmic HK FlgS (Wen et al. 2009). *Helicobacter pylori* produces urease up to 10% of its total protein content (Sachs et al. 2005). In animal models, urease-negative mutants are unable to colonize, further confirming the essential role of urease in gastric habitation (Eaton et al. 1991, Skouloubris et al. 1998, Mollenhauer-Rektorschek et al. 2002).

In several *Brucella* spp., urease activity is crucial to increase infectivity (Bandara et al. 2007, Sangari et al. 2007). Urea is also present at 3–10 mM concentrations in saliva and constitutes a nitrogen source for oral plaque microbiota (Mora and Arioli 2014). *Streptococcus salivarius* and *Actinomyces naeslundii* have been demonstrated to be urease positive (Morou-Bermudez and Burne 1999, Chen and Burne 2003), and viability of *S. salivarius* at extremely low pH increases in a urea concentration dependent manner (Chen et al. 2000).

Cellular remodeling to withstand acid stress

Synthesis of chaperones

Acidification leads to protein unfolding, denaturation, or damage as more amino acid residues become fully protonated (Goto et al. 1990). *Escherichia coli* has efficient chaperone-based mechanisms to protect proteins from acid-dependent denaturation and damage, especially in the periplasm (Fig. 2). HdeA and HdeB are two structurally related periplasmic chaperones that function in an ATP-independent manner and minimize protein aggregation due to high proton and chloride concentrations in the periplasm during acid stress (Fig. 2). They also assist in refolding the substrate after acid stress relief in *E. coli*, *Shigella flexneri*, *Brucella abortus*, and other species (Waterman and Small 1996, Gajiwala and Burley 2000, Valderas et al. 2005, Kern et al. 2007, Malki et al. 2008, Stull et al. 2018). The corresponding genes are usually part of the acid fitness island, where genes are induced in cells exposed to acid stress (Tucker et al. 2002).

In vitro, both chaperones are inactive dimers at neutral pH. At pH 2, the HdeA dimer rapidly converts into partially disordered, chaperone-active monomers. In its monomeric form, HdeA binds to proteins that were unfolded at low pH, effectively preventing them from aggregating (Tapley et al. 2009b). Upon return to neutral pH, HdeA slowly releases the bound proteins, allowing them to refold while minimizing the concentration of aggregation-prone intermediates (Tapley et al. 2009a). HdeA then converts back to its chaperone-inactive dimer.

In vivo, HdeA functions optimally in cells exposed to extreme acid stress of pH 2–3, and HdeB functions optimally at a moderately acidic pH of 4 (Dahl et al. 2015). The cytoplasmic chaperone Hsp31 (*hchA*) also contributes to acid stress resistance of stationary-phase *E. coli* cells (Mujacic and Baneyx 2006). Its transcription is controlled by sigma factor σ^S, but its exact function remains unclear. Some evidence indicates that cytoplasmic chaperones such as GroE, DnaK, and IbpB are upregulated in some Gram-negative bacteria under acid stress (Lund et al. 2014). Further experimental evidence is needed to determine the extent to which strong acidification of the cytoplasm leads to significant protein unfolding in the cytoplasm of these organisms.

Modifications of the cytoplasmic membrane

The cytoplasmic membrane is a major barrier to proton influx in acid exposed cells. To protect this membrane against acid damage, bacteria modulate its integrity, fluidity, and lipid composition. Under acid stress, the ratio of saturated to unsaturated fatty acids in the membrane increases (Yang et al. 2014). Furthermore, cyclopropane fatty acids (CFAs) are synthesized and incorporated into the membrane (Brown et al. 1997) (Fig. 2). In this process, unsaturated fatty acids are converted to CFAs by the postsynthetic addition of a methyl group from S-adenosyl-methionine to a double bond (Chang and Cronan 1999). An *E. coli* mutant lacking *cfa*, the gene encoding CFA synthase, is highly sensitive to acid stress, but this sensitivity can be partially overcome by an exogenous supply of CFAs (Chang and Cronan 1999). In addition, *E. coli* membranes lacking CFAs are more permeable to protons, suggesting that the presence of CFAs is important to maintain membrane integrity under acid stress (Shabala and Ross 2008). The transcription of the *cfa* gene is upregulated at low external pH, indicating that alteration of the cytoplasmic membrane fatty acid composition is an adaptive response mechanism (Chang and Cronan 1999). Changes in the cytoplasmic membrane composition in response to acid stress have also been observed in *Salmonella* and *Helicobacter* (Haque et al. 1996, Kim et al. 2005, Alvarez-Ordóñez et al. 2009).

In addition to modifications of fatty acids, the modification of lipid types has been described in acid-stressed bacteria. Common responses involve hopanoid formation and the introduction of hydroxyl groups into existing membrane lipids such as ornithine lipids, lipid A, or sphingolipids (Sohlenkamp 2019).

Synthesis of acid-shock proteins

Of the previously mentioned amino-acid decarboxylase systems, the Gad system contributes most to the survival of an *E. coli* population in extremely acidic conditions at or below a pH of 2.5 (Biase et al. 1999, Biase and Pennacchietti 2012). Such conditions however, as they are encountered in the human gastrointestinal tract,

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generally require adaptions on a global scale beyond amino-acid dependent AR systems (Kalantzi et al. 2006). Proteomic and microarray analyses have identified several candidates as acid shock proteins in cells exposed to acidified media (Blankenhorn et al. 1999, Audia et al. 2001, Stancik et al. 2002, Maurer et al. 2005) (Fig. 2). One prominent example is the periplasmatic Asr protein, which is crucial for survival in extreme acidity (Šeputienė et al. 2003). Expression of the *asr* gene can be induced by at least three transcriptional regulators, RstA, RcdA, and PhoB (Sužiedėlienė et al. 1999, Ogasawara et al. 2007, Shimada et al. 2012). Upon expression, Asr is post-translationally cleaved into an 8- and a 5-kDa protein, however, the biological significance of this processing is still unclear. Maximum induction occurs at pH 4.0–4.5, and *asr* null mutants display an acid-sensitive phenotype (Šeputienė et al. 2003). Other strongly induced potential acid shock proteins in *E. coli* have been identified in moderate acidity of pH 4.3–5.0 (Foster and Hall 1991), including several poorly characterized proteins and known heat shock proteins (Heyde 1990, Blankenhorn et al. 1999, Stancik et al. 2002, Maurer et al. 2005). Several acid shock proteins have also been identified in S. Typhimurium. For most acid shock proteins, the exact mechanisms of protection during acid shock remain elusive and require further characterization.

Another important cellular component that protects DNA from damage is the Dps protein. In *dps* mutants, acid-induced DNA damage is significantly increased (Choi et al. 2000, Jeong et al. 2008). In stationary-phase cells, Dps becomes the most abundant nucleoid component (180 000 molecules per cell) and causes compaction of DNA characteristics of this growth phase (Ali Azam et al. 1999). Dps also protects DNA from oxidative cleavage (Martinez and Kolter 1997). Both oxidative and acid stress are associated with the production of hydroxyl radicals ($OH^{\bullet}$) via Fenton-type reactions, which mainly lead to DNA single and double strand breaks by oxidizing the sugar and base moieties. Dps proteins inhibit formation of these toxic hydroxyl radicals via a multistep process of removing Fe(II) (Ceci et al. 2003, 2004). In conclusion, further proteome and transcriptome wide studies and an in-depth characterization of the most promising candidate proteins at various pH levels are needed to unravel the full spectrum of acid shock proteins in bacteria.

Acid-dependent chemotaxis

Beyond the above-mentioned AR mechanisms, chemotactic movements have been described in several bacterial species as a direct adaptation to physically avoid low pH environments (Fig. 2) (Kihara and Macnab 1981, Repaske and Adler 1981, Tohidifar et al. 2020). When bacteria sense acidic pH or weak organic acids such as acetate, they migrate to more desirable environments (Tsang et al. 1973, Tso and Adler 1974). For instance, weak acids induce prolonged tumbling in *E. coli* and *Salmonella* cells at pH 5.5 (Kihara and Macnab 1981). Moreover, a microarray study conducted at various pH levels revealed that flagellar and chemotaxis genes in *E. coli* are regulated in response to the pH (Maurer et al. 2005). Correspondingly, genes encoding the flagellar motility apparatus are upregulated in *H. pylori* at low pH (Merrell et al. 2003, Wen et al. 2003). The swimming behavior of bacterial cells is controlled by the direction of the flagellar motor, which alternates between clockwise and counterclockwise rotations (Berg 1974). If repellents such as acids are perceived, tumbling is enhanced by prolonged clockwise flagella rotation (Berg and Brown 1972, Silverman and Simon 1974).

In *E. coli*, alkaline and acidic environments are sensed by two methyl-accepting chemotaxis proteins (MCPs), the aspartate binding receptor, Tar, and the serine binding receptor, Tsr (Repaske and Adler 1981, Krikos et al. 1985, Khan et al. 1995). Using a fluorescence resonance energy transfer-based reporter, Yang and Sourjik (2012) reported a "push–pull" mode of action in which Tar initiates an attractant response to low pH and a repellent response to high pH, whereas Tsr exerts the opposing mode of action. This bidirectional response helps *E. coli* avoid extremely low or high pH by transitioning from acid-seeking to base-seeking and *vice versa* at specific pH inversion points. Another example for an MCP involved in pH taxis has been found in *H. pylori* (Croxen et al. 2006, Sweeney et al. 2012). Besides perception of the quorum-sensing molecule AI-2 (Rader et al. 2011), the chemotaxis receptor TlpB is indispensable for pH taxis and colonization of the gastric mucosa (Croxen et al. 2006). See below for further discussion of Tar, Tsr, and TlpB. To date, the strategy of avoiding acidic environments by chemotaxis has only been observed in *E. coli* and *H. pylori*. It remains unclear whether this strategy is specific to these species or whether it represents a universal stress response within bacteria. Further research is needed to clarify whether the number of flagella per cell is increased under low pH conditions, as upregulation of genes encoding the flagellar motility apparatus was observed.

Molecular mechanisms of pH-sensing

In the next chapters, we will discuss the molecular processes of pH-responsive receptors that are able to sense changes in pH outside and inside bacteria. In most cases, stimulus perception is coupled to transmembrane signaling.

Hybrid HK EvgS

The hybrid HK EvgS senses low external pH and transmits this signal to its cognate RR EvgA in *E. coli* (Utsumi et al. 1994, Eguchi and Utsumi 2014) (Figs. 3 and 4). The multidomain cytoplasmic part of EvgS comprises three signal transduction modules, including a transmitter domain (composed of an H-box containing dimerization and histidine phosphotransfer domain and a catalytic domain), a receiver domain, and a histidine-containing phosphotransfer (HPr) domain. The periplasmic part is connected via a cytoplasmatic linker and includes two Venus flytrap (VFT) domains (Perraud et al . 1998, Sen et al. 2017). Upon signal perception, the phosphoryl group at the first histidine residue (His721) in EvgS is transferred to an aspartate residue (Asp1009) in the receiver domain and then transmitted to another histidine residue (His1137) in the HPr domain. To complete the four-step His-Asp-His-Asp phosphorelay, the phosphoryl group is transferred to the aspartate residue (Asp52) in EvgA (Utsumi et al. 1996, Perraud et al. 1998, Tanabe et al. 1998, Kinoshita-Kikuta et al. 2015).

EvgS displays maximum activity at pH 5.0–5.5, and a leucine residue at position 152 in the first VFT domain is crucial for activation (Fig. 4) (Eguchi and Utsumi 2014). Sen et al. (2017) also elucidated the structure of the periplasmatic domain of EvgS. Additionally, the residues His226 and Pro522 are presumably involved in sensing acidity and signal transduction (Fig. 4).

In a proposed model, the stimulus is transduced from the VFT domains to the cytoplasmatic Per-Arnt-Sim (PAS) domain via structural rearrangements mediated by the region comprising Leu152, His226, and Pro522. In combination with further stimuli sensed in the cytoplasm, the structural change putatively triggers a weakening of subunit interactions in an inactive tight EvgS dimer. Subsequently, the loose dimer, which constitutes the active EvgS conformation, induces autokinase activity and initiates the described phosphorelay (Johnson et al. 2014, Sen et al. 2017). This model is further supported by the fact that several amino

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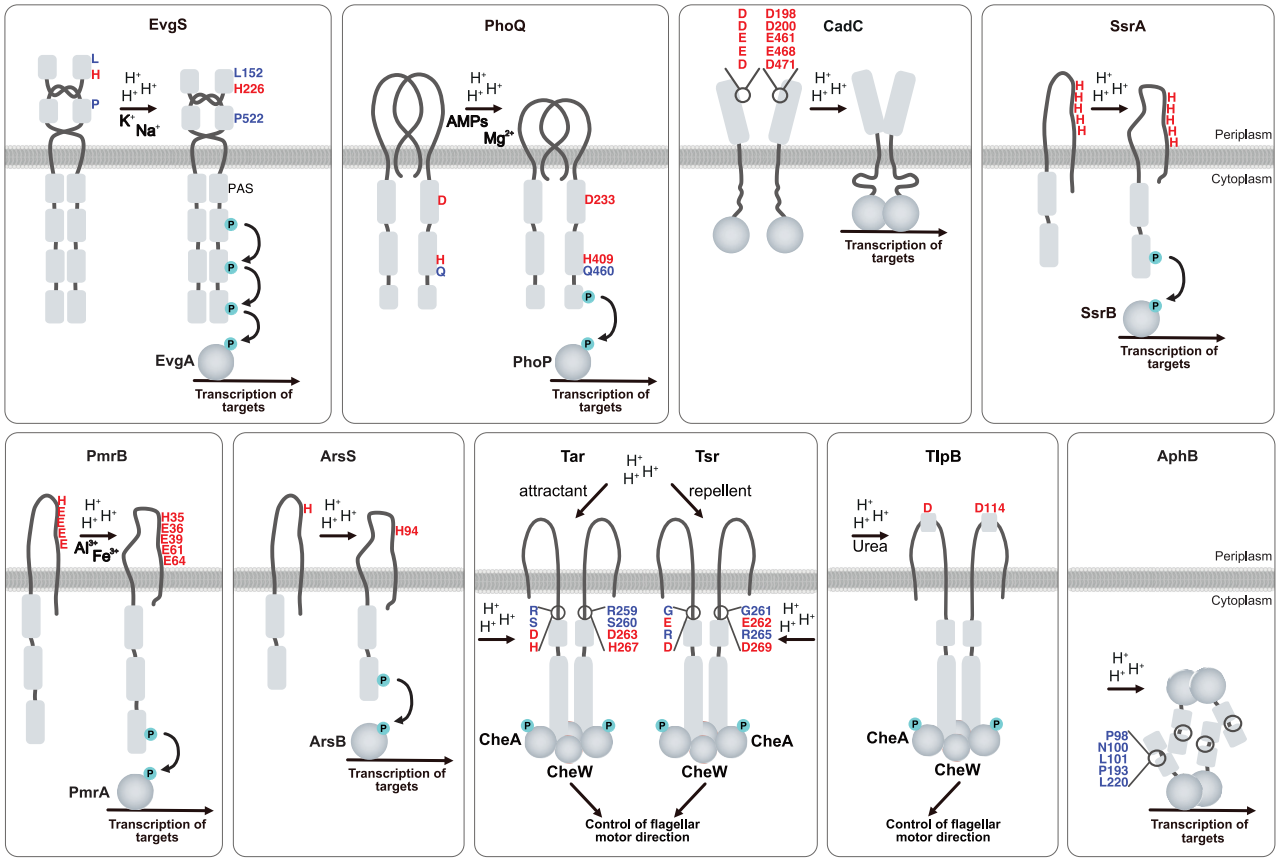

Figure 4. Schematic overview of the mechanisms of acid sensing of membrane-integrated and soluble pH sensors. The overall structure and mode of activation by low pH and various cations are indicated for the membrane-integrated sensors EvgS (Eguchi and Utsumi 2014, Hicks et al. 2015, Choi and Groisman 2016, Sen et al. 2017), PhoQ (Choi and Groisman 2016), CadC (Haneburger et al. 2011), SsrA (Mulder et al. 2015), PmrB (Perez and Groisman 2007), ArsS (Müller et al. 2009), Tar and Tsr (Umemura et al. 2002), as well as TlpB (Sweeney et al. 2012) and for the soluble regulator AphB (Taylor et al. 2012). Amino acid residues of the regulators are marked in red when described to be important for the recognition of low pH. For SsrA, only five histidine residues in the C-terminal end are described to be important for the recognition of low pH. Amino acid residues marked in blue are connected with the adaptation to low pH. Low pH is indicated via "H^+." AMPs, antimicrobial peptides. PAS, Per-Arnt-Sim domain.

acid replacements, which constitutively activate EvgS, have been identified in the PAS domain. These amino acid replacements might mimic the rearrangement from an inactive tight dimer to a more dissociated conformation, which constitutes the active state (Johnson et al. 2014).

In addition to the required mild acidity, alkali metals with a concentration of at least 150 mM are necessary to activate the EvgS/EvgA system (Eguchi and Utsumi 2014). To sense acidic pH, both the periplasmatic region and the PAS domain are required, while alkali metals (e.g. K^+) can be sensed by either domain individually (Eguchi and Utsumi 2014, Johnson et al. 2014). Beyond these findings, EvgS is active in mildly acidic environments under aerobic but not in anaerobic growth conditions. The EvgS PAS domain is hypothesized to act as an intermediate redox switch that transmits information from the periplasm to the cytoplasmic catalytic core, depending on the oxidation state of ubiquinone in the electron transport chain (Inada et al. 2021).

Due to substantial amino acid polymorphisms across different *E. coli* isolates, EvgS displays large variability in its pH-sensing capacity, including pH nonresponsive variants (Roggiani et al. 2017). The kinase forms clusters in *E. coli* (Sommer et al. 2013). Once EvgA is phosphorylated, it activates expression of two genes coding for regulators of the Gad system, *ydeO* and *gadE* (Ma et al. 2004, Itou et al. 2009). EvgA acts as a homodimer; however, it may also form heterodimers with RcsB (Johnson et al. 2011, Pannen et al. 2015). Moreover, the EvgS/EvgA system regulates the expression of multiple multidrug efflux pumps, including EmrYK and MdtEF (formerly YhiUV) (Kato et al. 2000, Nishino and Yamaguchi 2001, Eguchi et al. 2003).

HK PhoQ

Together with EvgS, PhoQ is responsible to transmit the activating signal of the Gad system in *E. coli*. In addition, the kinase has also been extensively studied in S. Typhimurium. The amino acid sequences of PhoQ in both bacterial species are 86% identical (Kasahara et al. 1992). As shown in Fig. 4, the bifunctional PhoQ is membrane-integrated and comprises a periplasmic sensor domain, a transmembrane TM 4-helix bundle, cytoplasmic HAMP domain, DHp domain, and a kinase domain (Goldberg et al. 2010). Under physiological conditions, PhoQ acts as a homodimer (Goldberg et al. 2008). Activation by various stimuli leads to autophosphorylation of PhoQ at a conserved histidine residue and subsequent phosphotransfer to the RR PhoP (Groisman et al. 1989, Miller et al. 1989). Under noninducing conditions, PhoQ displays phosphatase activity and dephosphorylates PhoP (Gunn and Miller 1996, Shin and Groisman 2005).

In *E. coli*, the phosphorelay between PhoQ and PhoP is fine-tuned by the small membrane protein SafA, which accelerates PhoQ autophosphorylation and accumulation of phosphorylated PhoP by direct interaction between its C-terminal domain and

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the periplasmic sensor domain of PhoQ (Ishii et al. 2013). Transcription of *safA* itself is under the control of EvgA and thus connects the PhoQ/PhoP to the EvgS/EvgA system (Masuda and Church 2003, Eguchi et al. 2007, Itou et al. 2009). This connection to the EvgS acid-sensor is crucial, as direct pH-sensing has not been demonstrated for PhoQ in *E. coli*. Similar to *E. coli*, an additional horizontally acquired gene (*ugtL*) is needed to enhance autophosphorylation of PhoQ in S. Typhimurium (Choi and Groisman 2017). Both UgtL and SafA are required for the response of the PhoQ/PhoP system in mildly acidic conditions but not for sensing low Mg^{2+} levels and also do not affect phosphotransfer from PhoQ to PhoP (Groisman et al. 2021). SsrB (see below) is another factor involved in S. Typhimurium's response to acid stress mediated by PhoQ/PhoP, as it binds to the *ugtL* promoter region and counteracts the repressing effects of H-NS (Choi and Groisman 2020).

In *E. coli*, the PhoQ/PhoP system is regulated at the post-transcriptional level by two Hfq-dependent small RNAs, MicA and GcvB. Both independently base-pair with the translation initiation region of the *phoPQ* mRNA, which impairs ribosomal binding and promotes transcript degradation (Coornaert et al. 2010, 2013). In addition, the PhoQ/PhoP system also involves a negative feedback inhibition loop via the small-membrane protein MgrB (formerly YobG). PhoP initiates expression of *mgrB*, and MgrB in turn inhibits the kinase activity of PhoQ (Lippa and Goulian 2009, Salazar et al. 2016). Once the PhoP RR is phosphorylated, it transcriptionally controls hundreds of target genes (5% of all genes) in S. Typhimurium (Belden and Miller 1994, Soncini et al. 1996, Zwir et al. 2005, Harari et al. 2010, Zwir et al. 2012). The PhoQ/PhoP system reaches maximum activity around pH 5.0, which corresponds to the pH of the macrophage phagosomes (Alpuche Aranda et al. 1992, Rathman et al. 1996, Bearson et al. 1998). PhoP-dependent gene regulation itself depends on phosphorylated PhoP amounts, which are affected by numerous factors including PhoP acetylation, feedback loops, proteolysis by ClpSAP, the Mg^{2+} transporter MgtA, $EIIA^{Ntr}$, and others, as reviewed in Groisman et al. (2021). In *E. coli*, the PhoP regulon includes several AR genes, including *hdeD*, *hdeAB*, and the central Gad system regulators *gadW* and *gadE* (Belden and Miller 1994, Zwir et al. 2005, Eguchi et al. 2011).

Only in S. Typhimurium, protons have been shown to be directly sensed by PhoQ (Prost et al. 2007, Hicks et al. 2015, Choi and Groisman 2016). Nuclear magnetic resonance studies show that the PhoQ periplasmic sensor domain undergoes a conformational change at mildly acidic pH levels and involves a network of residues surrounding His157 (Prost et al. 2007). Moreover, α-helices 4 and 5 in this domain may act as potential pH-responsive structural elements altering the dynamic behavior of PhoQ in an acid-dependent manner. Introducing a nonphysiological disulfide bond between the substitutions Trp104Cys and Ala128Cys restrains flexibility in α-helices 4 and 5 of the periplasmic domain and subsequently impairs PhoP-mediated gene expression during acid stress (Hicks et al. 2015). However, those results have been challenged by the findings of Choi and Groisman (2016), who discovered three residues in the cytoplasmic PhoQ domain (Asp233, His409, and Gln460) that are indispensable for pH sensing (Fig. 4). Their results also indicate that PhoQ senses acidic pH exclusively in the cytoplasm, as *in vivo* experiments demonstrate that mutants lacking the PhoQ transmembrane and periplasmatic regions, as well as chimeras with the EnvZ periplasmatic domain, still respond to acidity. Nevertheless, it remains elusive whether protons itself are sensed by the critical amino acids in the cytoplasm or rather a different stimulus which is triggered by intracellular acidification (Choi and Groisman 2016, Groisman et al. 2021).

In addition to sensing low pH, PhoQ in S. Typhimurium and *E. coli* can sense a wide variety of other signals, including divalent cations (Mg^{2+}, Mn^{2+}, and Ca^{2+}), hyperosmotic stress, the periplasmatic redox state, and acetate (Groisman et al. 2021). Antimicrobial peptides (AMPs) and long-chain unsaturated fatty acids can be bound by the periplasmic PhoQ domain in *Salmonella* (Bader et al. 2003, Shprung et al. 2012, Viarengo et al. 2013, Carabajal et al. 2020). Protons and AMPs are presumably sensed via distinct signaling mechanism as they have additive effects on PhoQ activation, whereas divalent cations and AMPs compete for binding in the periplasmic domain (Bader et al. 2005, Prost et al. 2007, Hicks et al. 2015). Remarkably, in the nonpathogenic strain *Salmonella bongori*, Mg^{2+} and AMPs can be sensed, but mildly acidic pH levels do not trigger PhoP-dependent gene expression, presumably because the crucial residues involved in cytoplasmic pH sensing (Fig. 4) are not conserved in *S. bongori* (Choi and Groisman 2016). In *E. coli*, only His409 but not Asp233 and Glu460 are conserved, which supports the idea that protons are not directly, but rather indirectly sensed via EvgS and SafA. A further critical residue for signaling in *E. coli* PhoQ is Asn202 in the second transmembrane helix. A substitution with other hydrophobic residues after exposure to Mg^{2+} results in disrupted signal propagation and failed expression of PhoP-activated genes (Goldberg et al. 2010).

ToxR-like receptor CadC

Unlike EvgS and PhoQ, CadC belongs to the one-component signal transduction systems (ToxR-like family) of transcriptional regulators. These low-copy membrane-integrated receptors combine sensory and transcriptional regulatory functions in one polypeptide (Miller et al. 1987). ToxR controls virulence in *V. cholerae*. Nevertheless, other members of this family contribute to virulence, acidic stress response and biofilm and type VI pilus formation (DiRita and Mekalanos 1991, Merrell and Camilli 2000, Stelzer et al. 2006). These receptors have a conserved modular composition: a C-terminal periplasmic sensor domain that senses external stimuli and a single transmembrane helix to transduce the signal to the N-terminal cytoplasmic winged helix-turn-helix DNA-binding domain, which then regulates gene expression. This signal transduction is independent from chemical modifications (Miller et al. 1987).

In 2011, the crystal structure of the periplasmatic domain of *E. coli* CadC was solved (Eichinger et al. 2011). This sensory domain consists of two subdomains: the N-terminal subdomain comprises a mixture of parallel and antiparallel β-sheets and a three-helix-bundle, and the C-terminal subdomain is a pure bundle of 11 α-helices. Distinct amino acids within the periplasmic sensory domain of CadC are responsible for dimerization, pH sensory function, and feedback inhibition by cadaverine. To detect alterations in external pH, a cluster of negatively charged amino acids, namely Asp198, Asp200, Glu461, Glu468, and Asp471, are essential (Fig. 4). This negatively charged patch extends across the N- and the C-terminal subdomains. It is proposed that protonation of this cluster at the dimer interface within the periplasmic domain of CadC enables conformational changes resulting in dimerization and signal transduction (Eichinger et al. 2011, Haneburger et al. 2011, 2012, Buchner et al. 2015, Schlundt et al. 2017).

HK SsrA

The HK/RR system SsrA/SsrB of S. Typhimurium controls, amongst others, the virulence genes encoded in the *Salmonella pathogenicity* island 2 (SPI-2), which are required for intracellular survival in host cells. SsrA signaling requires acidification of

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the cytoplasm of S. Typhimurium due to the acidic environment of the macrophage vacuole of the host in which the bacteria reside. Response to external acidification is integrated through SsrA, whereas acidification of the cytoplasm affects the RR SsrB. As shown in Fig. 4, five histidine residues in the C-terminal end of the periplasmic sensor domain are important for the full acid-responsiveness of SsrA (Mulder et al. 2015). Yet, an SsrA variant lacking the important histidine residues of the periplasmic domain shows basal activity at neutral pH, similar to the activity of wild type. As no single histidine is responsible for the full acid activation of SsrA, Mulder et al. (2015) concluded that the concerted action of multiple histidine residues is needed, as with the diphtheria toxin T domain (Perier et al. 2007).

Acidification of the cytosol induces conformational changes in SsrB, increasing its affinity to bind to DNA (Liew et al. 2019). This pH-dependent DNA-binding of SsrB has been shown by atomic force microscopy and single-particle tracking assays. Binding of PhoP or OmpR is not pH-sensitive; thus, acid-dependent DNA binding of SsrB is not an intrinsic property of transcriptional regulators (Liew et al. 2019). Nevertheless, S. Typhimurium acidifies its cytoplasm in an OmpR-dependent manner by repressing the CadC-regulated *cadBA* operon (Chakraborty et al. 2015). Moreover, the HK/RR system EnvZ/OmpR upregulates the SsrA/SsrB system upon stimulation by acid stress by directly activating the *ssrAB* promoter (Lee et al. 2000, Garmendia et al. 2003, Fass and Groisman 2009, Chakraborty et al. 2015, Liew et al. 2019). The EnvZ/OmpR system integrates acidic pH, absence of Ca^{2+} and low osmolarity (Garmendia et al. 2003) and is required for the replication and survival (Lee et al. 2000) of *Salmonellae* in macrophages. It also is necessary for full virulence in mice (Dorman et al. 1989, Chatfield et al. 1991). Overall, instead of recovering from acid stress, S. Typhimurium use the acidification of its cytoplasm as a signal to activate on the one hand PhoQ (Choi and Groisman 2016) and on the other hand its pathogenesis via SsrB that upregulates the expression and secretion of SPI-2 effector proteins (Kenney 2019).

HK PmrB

In addition to the SsrA/SsrB and PhoQ/PhoP systems, S. Typhimurium also possess the PmrB/PmrA HK/RR system, which is involved in perceiving extracellular acidic conditions. The HK PmrB is activated in mildly acidic conditions (pH 5.8), and the RR PmrA promotes transcription of several genes (Perez and Groisman 2007). PmrB and PmrA are required for various pathways, such as virulence in mice (Gunn et al. 2000), infection of chicken macrophages (Zhao et al. 2002), growth in soil (Chamnongpol et al. 2002), resistance to the cationic peptide antibiotic polymyxin B (Roland et al. 1993), and resistance to Fe^{3+} (Wösten et al. 2000) and Al^{3+} (Nishino et al. 2006). For full acid-responsiveness of PmrB, a histidine and four glutamic acid residues in the periplasmic domain of PmrB are required (Fig. 4) (Perez and Groisman 2007). Submillimolar levels of extracellular Fe^{3+} or Al^{3+} are perceived directly by PmrB (Wösten et al. 2000), and low concentrations of extracellular Mg^{2+} (Soncini and Groisman 1996) trigger transcription of PmrA-activated genes. The activation of PmrA by low extracellular Mg^{2+} levels occurs via activated PhoQ, which directly senses the extracellular Mg^{2+}, followed by phosphorylation of its cognate RR PhoP, which then activates transcription of *pmrD*. PmrD then promotes phosphorylation of PmrA and inhibits PmrB-promoted dephosphorylation of phosphorylated PmrA (Kox et al. 2000, Kato and Groisman 2004). Multiple signals are integrated into two-component systems similar to the signal transduction cascades in eukaryotes.

HK ArsS

The human gastric pathogen *H. pylori* is extremely well-adapted to the fluctuating low-pH conditions in the human stomach. This adaptation is mainly mediated via the NH_3-producing enzyme urease, which is essential for maintaining neutral pH in both the cytoplasm and periplasm. Urease-independent mechanisms are likely to contribute to acid adaptation. In response to low pH, hundreds of genes are regulated, including the urease gene cluster, as identified by four independent studies performing genome-wide transcriptional profiling (Merrell et al. 2003, Wen et al. 2003, Bury-Moné et al. 2004, Pflock et al. 2006).

The ArsS/ArsR HK/RR system is the main regulator for the acid response. An acidic pH of 5.0 triggers autophosphorylation of ArsS, followed by the subsequent phosphorylation of its cognate RR, ArsR. Phosphorylated ArsR then acts both as an activator and repressor of pH-responsive genes (Pflock et al. 2006). To perceive low pH, the histidine residue at position 94 of ArsS is crucial (Fig. 4). Moreover, depending on the degree of protonation of several acidic amino acids in the periplasmic domain, ArsS can adopt different conformations resulting in different activation states to allow a gradual transcriptional response to moderate low pH conditions (pH 5.0). Müller et al. (2009) concluded that pH sensing by ArsS does not rely exclusively on protonation of histidine residues but rather on protonatable amino acids other than histidine that contribute substantially to acidity. The acid-induced upregulation of the urease gene cluster requires the sensor kinase ArsS but not phosphorylation of the RR ArsR. Although ArsR phosphorylation increases expression of the urease gene cluster, it is not required for acid survival.

Chemoreceptors Tar and Tsr

Tar and Tsr are required to navigate *E. coli* to a desired intermediate pH level and to physically avoid habitats with extreme pH levels. Both receptors form clusters that associate with the adaptor protein CheW and the HK CheA, resulting in initiation of a signal transduction cascade and ultimately in regulation of the flagellar motor to navigate the cell to desired environments (Maddock and Shapiro 1993, Kentner et al. 2006, Hazelbauer et al. 2008). Both MCPs can sense internal as well as external pH. Ambient pH is sensed by the periplasmatic domains, and residues for sensing the internal pH have been identified in cytoplasmic regions (Krikos et al. 1985). By characterizing chimeras, residues in Tsr (Gly261, Glu262, Arg265, and Asp269) and Tar (Arg259, Ser261, Asp263, and His267) have been identified as crucial in the chemotactic response to alterations of the internal pH (Fig. 4). Interchanging these residues located in the linker region, which connects the second transmembrane helix to the first methylation helix, affects the pH response of Tar and Tsr. While a replacement of Arg259 by the corresponding residue of Tsr reversed the swimming behavior, a substitution of Asp263 or His267 only altered the time course of pH response (Umemura et al. 2002).

Especially for Tsr but also for Tar, adaptive receptor methylation was indicated to fine-tune the strength of the pH response due to a weakened response upon methylation of either receptor. At low pH levels, Tsr is the dominant receptor as it has fewer methylated glutamyl residues and guides the cells toward higher pH values, whereas at alkaline pH levels, Tar is less methylated and dominates the pH response. Moreover, the pH inversion point is affected by culture density-dependent alterations in Tar and Tsr

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expression levels (Yang and Sourjik 2012). At high cell densities, the preferential pH point is shifted toward lower pH units as the ratio of Tar/Tsr increases, presumably to avoid nutrient-depleted regions of high cell numbers, which were demonstrated to display more alkaline pH values (Salman and Libchaber 2007, Kalinin et al. 2010, Yang and Sourjik 2012).

Chemoreceptor TlpB

Similar to Tar and Tsr in *E. coli*, TlpB is the crucial receptor for pH taxis in *H. pylori*. In a mouse infection model, *tlpB* mutants were shown to be pH nonresponsive and defective for colonization (Croxen et al. 2006). An atomic resolution structure of the periplasmic domain has been solved by Sweeney et al. (2012), showing that TlpB forms dimers via extracellular urea-binding PAS-domains and contains two transmembrane regions per subunit and a cytoplasmic HAMP domain.

Asp114 in the extracellular PAS-domain of the TlpB dimer is proposed to be the key titratable residue for signaling, while urea acts as a cofactor for acid sensing. In a proposed model, urea binding is weakened via protonation of Asp114 at low pH levels and a structural rearrangement within the PAS-domain is induced. Moreover, urea protects the TlpB periplasmic domain against thermal denaturation in a pH-dependent manner. Thereby, Asp114 accepts two hydrogen bonds from the amide nitrogen atoms of urea at neutral pH and stabilizes the PAS domain. This interaction, however, is disrupted or weakened in acidic environments due to protonation of Asp114. This in turn may cause a loosening of urea binding and partial unfolding of the PAS-domain. Subsequent movement of TM2 relative to TM1 could affect the phosphorylation state of CheA (Sweeney et al. 2012). Little is known about the transcriptional regulation of *tlpB*. The only thus far identified protein to bind the promoter region of *tlpB* is the orphan RR HP1043 (Delany et al. 2002).

Transcriptional regulator AphB

In addition to membrane integrated proteins, also cytosolic regulators like AphB have been described to harbor pH-sensing residues. AphB is an important regulator in several *Vibrio* spp. for adapting to environmental conditions and pathogenicity. AphB belongs to the family of LysR-type transcriptional regulators (LTTR), one of the largest families of regulators in prokaryotes (Maddocks and Oyston 2008). Together with the quorum sensing-regulated activator AphA, $AphB_{Vc}$ activates the virulence cascade via *tcpPH* expression of the human pathogen *V. cholerae* (Kovacikova and Skorupski 1999). $AphB_{Vc}$ also regulates several genes involved in pH regulation like CadC (Kovacikova et al. 2010). In *V. cholerae*, low pH and anaerobiosis are the two environmental signals leading to activation of AphB (Kovacikova et al. 2010).

Taylor et al. (2012) solved the crystal structure of full-length $AphB_{Vc}$, showing that AphB forms a tetramer by dimerization of dimers like other members of the LTTR family. AphB consists of an N-terminal winged helix DNA-binding domain and a C-terminal regulatory domain. The DNA-binding domain is formed by three α-helices (α1–α3) followed by two β-strands that form the wing. The C-terminal regulator domain has two subdomains, RD-I and RD-II, connected by two extended antiparallel β-strands. Their interface harbors a putative ligand-binding pocket formed by hydrophobic and polar amino acids. A total of five residues in the ligand-binding pocket of AphB can render the activity of the protein to nearly constitutive activity, with respect to pH and oxygen, when they are replaced by negatively charged amino acids. These residues are Pro98, Asn100, and Leu101, which are located in the loop between β3 and α5; residue Pro193, located in the loop between β7 and β8, and residue Leu220 (Fig. 4).

$AphB_{Vc}$ was crystallized alone (wild type) and bound to the DNA (Asn100Glu variant). A comparison of these two structures shows that $AphB_{Vc}$ undergoes a major conformational change to alter the position of the DNA-binding domain (Taylor et al. 2012). These findings also have been observed for AphB in *Vibrio vulnificus*, where $AphB_{Vv}$ affects the expression of more than 10% of the genome, including *cadC* (Jeong and Choi 2008). The crystal structure of the regulatory domain of $AphB_{Vv}$ has been solved and shows high similarity to $AphB_{Vc}$. The putative ligand binding pocket consists of 12 amino acids (Pro98, Asp100, Leu101, Ser128, Val144, Asp162, His192, Pro193, Leu220, Pro237, Met240, and Arg262). An additional small cavity on the backside of the regulatory domain also has been identified (Lys103, Arg104, Asn221, Asn224, Met240, and Tyr244). Further study is needed to determine if it also contributes to ligand binding (Park et al. 2017).

Phylogenetic occurrence of the Gad, Adi, Cad, and Orn systems in the bacterial kingdom

Enteropathogenic bacteria (e.g. *Escherichia*, *Salmonella*, and *Yersinia*) generally possess several enzyme-based AR systems to survive in highly acidic environments, such as the stomach, and mild acidic ones, such as the caecum and ascending colon, however, this is not the usual case. Overall, the number, architecture and the complexity of regulation of the four main systems, Gad, Adi, Cad, and Orn, vary amongst bacterial species and often reflect an adaptation to their (acidic) habitat (Zhao and Houry 2010). The most widespread systems are the Gad and Orn systems, which co-occur in many Proteobacteria and Firmicutes. Bacterial species such as *Escherichia*, *Shigella*, *Citrobacter*, *Hafnia*, and *Serratia* have all four enzyme-based AR systems; *Salmonellae* have the Adi, Cad, and Orn systems; whereas the Gram-positive *Lactococcus* (Amachi et al. 1998, Sanders et al. 1998, O'Sullivan and Condon 1999) and *Listeria* (Davis et al. 1996, Cotter et al. 2001a, Arcari et al. 2020) possess the Gad system only (Zhao and Houry 2010). Species, like *Vibrio* spp. that generally are more sensitive to acid (Brenneman et al. 2014, Brameyer et al. 2020), contain only the Cad and Orn systems.

The different number of enzyme-based AR systems in the various bacteria is also reflected by the number of bacteria required to cause infection in humans. For example, *Shigella* spp. have a uniquely low infective dose of only 10–100 *Shigella* cells, whereas *Vibrio cholerae* infection requires about 10^8 organisms and *Salmonella* infection requires 10^5 to 10^7 (Table 1). *Escherichia* and *Shigella* spp. are the most acid-resistant neutralophilic bacteria. The *E. coli* K12 lab strains DH5α and MC4100 are as acid resistant as naturally occurring *E. coli* isolates (Gorden and Small 1993).

Pseudomonas spp. contain only the Orn system (Brameyer et al. 2022), which generally plays a minor role in AR, but are additionally equipped with deiminase and urease systems to withstand acidic environments.

The Gad system plays an important role for commensal and pathogenic bacteria, such as *E. coli* and *S. flexneri* (Waterman and Small 2003, Bhagwat and Bhagwat 2004), and it was recently identified in *Yersinia* (Foster 2004, Biase and Pennacchietti 2012, Pennacchietti et al. 2018, Brameyer et al. 2022), *Clostridium* (Villarreal et al. 2000, Tennant et al. 2007, Biase and Pennacchietti 2012), *Listeria monocytogenes* (Cotter et al. 2001a, 2001b), *Lactobacillus brevis* (Gong et al. 2019) and *Lactococcus lactis* (Sanders et al. 1998, O'Sullivan and Condon 1999). See Table 2 and Fig. 5 for details

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Table 1. Infective doses of food-borne pathogens.

Pathogen	Infective dose	Reference
Enterohemorrhagic *E. coli* (EHEC)	< 50	Tilden et al. (1996)
Brucella spp.	< 10	Román et al. (2013)
Shigella spp.	10–100	Sansonetti (2001)
Salmonella spp.	10^5–10^7	D'Aoust et al. (2001)
Vibrio cholerae	10^4–10^8	Cash et al. (1974)
Yersinia enterocolitica	10^4–10^9	Bottone (1997)
Campylobacter jejuni	500–800	Robinson (1981)
Clostridium perfringens	10^7	Brynestad and Granum (2002)
Listeria monocytogenes	> 100 bacteria/g*	Swaminathan (2001)

*Number in contaminated food responsible for foodborne human cases.

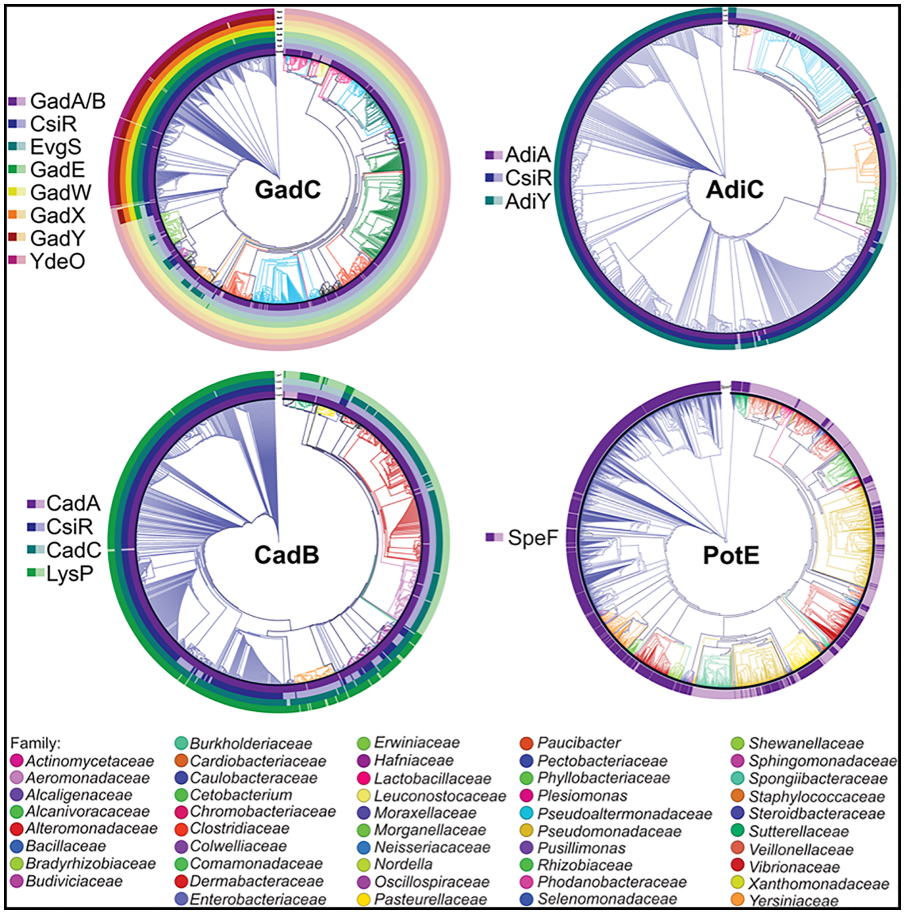

Figure 5. Phylogenetic trees of the *E. coli* antiporters GadC, AdiC, CadB, and PotE and co-occurring regulators within the bacterial kingdom. The protein sequences of 1112 of *E. coli* GadC homologs (upper left) were aligned, and a phylogenetic tree was generated. The presence of different regulatory components CsiR, EvgS, GadE, GadW, GadX, GadY, and YdeO and the decarboxylases GadA and GadB (GadA/B) is indicated by solid colors in the respective ring layer. Translucent colors represent components that were not detected. The protein sequences of 756 *E. coli* AdiC homologs (upper right) were aligned, and a phylogenetic tree was generated. The presence of the different regulatory components CsiR and AdiY as well as the decarboxylase AdiA is indicated by solid colors in the respective ring layer. If these components were not found, the colors are translucent. The protein sequences of 857 *E. coli* CadB homologs (lower left) were aligned, and a phylogenetic tree was generated. The presence of the different regulatory components CsiR, CadC, and LysP as well as the decarboxylase CadA are indicated by solid colors in the respective ring layer. Translucent colors represent components that were not detected. The protein sequences of 2577 *E. coli* PotE homologs (lower right) were aligned, and a phylogenetic tree was generated. The presence of the decarboxylase SpeF is indicated by solid colors in the respective ring layer. See legend of Table 2 for more details. Each phylogenetic tree is shown as a circular cladogram. For each phylogenetic tree the branches of the tree were colored according to the family of the organisms containing a GadC, AdiC, CadB, or PotE homolog, respectively.

on these and other bacteria discussed in this section.

Regulation of the Gad system has been studied in great detail in *E. coli* (see above). The regulators responsible for the sophisticated regulation of the Gad, Adi, and Cad systems of *E. coli* (Fig. 3) are present mainly in the *Enterobacteriaceae* family. The central regulator of the Gad system, GadE, is found in *Escherichia* and *Shigella*, as well as other *Enterobacteriaceae* species, such as *Citrobacter freundii* and *Kluyvera ascorbate* (Fig. 5) (Brameyer et al. 2022). The carbon-

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Table 2. Occurrence of the main components of the Gad, Adi, Cad, and Orn system within selected species of the proteobacteria and firmicutes. Homologs of the components of the Gad, Adi, Cad, and Orn system in the selected species were identified based on the proteins from *E. coli* MG1655. Phylogenetic distribution of the Gad, Adi, Cad, and Orn system is presented in Fig. 5. "x" indicates presence of a homolog of the proteins GadA/B, GadC, CsiR, EvgS, GadE, GadW, GadY, YdeO, AdiA, AdiC, AdiY, CadA, CadB, CadC, LysP, SpeF, and PotE. This table is modified and extended from Brameyer et al. (2022) for the occurrence of the Orn system. Briefly, homologs of the Orn system were identified as described previously (Brameyer et al. 2022) with the modified expect thresholds for the Protein BLAST search ($e < 10^{-40}$ for PotE and $e < 10^{-170}$ for SpeF) resulting in the identification of 2577 PotE homologs. These are present with Proteobacteria, such as *Enterobacteriaceae* (36.4%, with 20.0% belonging to *Escherichia*); *Pseudomonaceae* (17.6%), *Vibrionaceae* (15.0%), and *Burkholderiaceae* (4.0%); and within Firmicutes, such as *Streptococcaceae* (5.2%), *Yersiniaceae* (4.3%), *Lactobacillaceae* (1.2%), and *Clostridaceae* (0.5%).

		GadA/B	GadC	CsiR	EvgS	GadE	GadW	GadX	GadY	YdeO	AdiA	AdiC	AdiY	CadA	CadB	CadC	LysP	SpeF	PotE
Proteobacteria	*Escherichia coli*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Escherichia albertii	x	x	x		x	x	x	x		x	x	x	x	x	x	x	x	x
	Shigella boydii	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x
	Shigella flexneri	x	x	x	x	x	x	x	x	x	x	x	x					x	x
	Salmonella enterica			x							x	x	x	x	x	x	x	x	x
	Citrobacter freundii	x	x	x		x					x	x	x	x	x		x	x	x
	Hafnia alvei	x	x	x	x						x	x		x	x	x	x	x	x
	Serratia fonticola	x	x		x						x	x		x	x	x	x	x	x
	Yersinia spp.	x	x		x						x	x		x	x	x	x	x	x
	Stenotrophomonas maltophilia										x	x							
	Edwardsiella tarda													x	x	x	x	x	x
	Pseudomonas fluorescens																	x	x
	Morganella morganii	x	x											x	x		x	x	x
	Brucella spp.	x	x																
	Vibrio parahaemolyticus													x	x	x		x	x
	Vibrio cholerae													x	x	x		x	x
	Vibrio campbellii													x	x	x		x	x
Firmicutes	*Clostridium perfringens*	x	x														x		
	Lactococcus lactis	x	x														x		
	Listeria monocytogenes	x	x														x		
	Enterococcus faecium	x	x																
		Gad system									**Adi system**			**Cad system**				**Orn system**	

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induced starvation transcriptional regulator CsiR is a member of the GntR-family and mainly occurs in γ-proteobacteria. A total of 30.3% of species containing a GadC homolog possess an CsiR homolog (Fig. 5). Comparison of GadB homologs from Gram-positive and Gram-negative enteric bacteria shows that 84 residues (18% of the *E. coli* GadB sequence) are strictly conserved, including residues at critical positions in or near the active site of *E. coli* GadB or at sites of conformational change. Moreover, eight residues that are shared with group II decarboxylases may serve as signatures for bacterial glutamate-dependent AR (Biase and Pennacchietti 2012).

Besides using ureases to encounter acidic threats, *Brucella microti* also uses the Gad system to increase AR and oral infection in mice (Occhialini et al. 2012). An intact *gadBC* operon helps *B. microti* to survive in acidic soils and supports intracellular survival, such as within host macrophages (Audic et al. 2009). In 2015, Damiano et al. (2015) investigated the functionality of the Gad system among a collection of strains belonging to the *Brucella* genus and showed that not all species could export GABA and survive at extremely low pH levels; the authors thus concluded that the Gad system may reflect the adaptive evolution of *Brucella* spp.

In addition to the variations in bacterial AR systems, the architecture of individual AR systems also can vary. For example, the number of glutamate decarboxylases (up to three) and number of antiporters (up to two) varies in some bacteria, such as *Edwardsiella tarda*, *L. brevis*, and *L. monocytogenes* (Cotter et al. 2001a, 2001b, Cotter et al. 2005, Karatzas et al. 2010, Feehily and Karatzas 2013). Moreover, *E. coli* (Smith et al. 1992) and *S. flexneri* (Waterman and Small 2003) each contain two glutamate decarboxylases, GadA and GadB (Feehily and Karatzas 2013).

The Adi and Cad systems are not as widely distributed as the Gad system and occur mainly within γ-proteobacteria in the *Enterobacteriaceae*, *Lysobacteraceae*, *Yersiniaceae*, and *Hafniaceae* families (Brameyer et al. 2022) (see Table 2 and Fig. 5 for details).

However, *S.* Typhimurium is one well-studied example that harbors the Adi (Kieboom and Abee 2006), Cad (Lee et al. 2007), and Orn systems (Viala et al. 2011) (Table 2 and Fig. 5), and its virulent strains are more acid tolerant than its nonvirulent strains (Wilmes-Riesenberg et al. 1996, 1997, Berk et al. 2005). These three systems confer AR in different ways in this serovar. Specifically, the Adi system is effective at extremely acidic pH levels but ineffective during growth at moderately acidic pH levels. The Orn system improves growth at moderately acidic pH levels in the absence of oxygen, but it plays a minor role in survival. The Cad system has a broad range of actions and confers both to significant survival at pH 2.3 and to growth improvement at pH 4.5 in an oxygen-independent manner (Viala et al. 2011). Unlike *E. coli*, in which transcription of *cadC* is constitutive, in *S.* Typhimurium, *cadC* expression is induced by mildly acidic pH levels and by lysine (Lee et al. 2007). Instead of recovering from acid stress, *S.* Typhimurium uses acidity as a signal to control pathogenesis via the action of OmpR, which represses the *cadC /cadBA* module, thereby preventing neutralization of the bacterial cytoplasm (Chakraborty et al. 2015, Kenney 2019). Both the Cad and Orn systems enhance *S.* Typhimurium's acid tolerance. The *spe* genes (*speB, speC, speE*, and *speF*) have been shown to contribute to intracellular survival and bacterial replication for 18 h in human epithelial cells and for 21 h in macrophages (Espinel et al. 2016).

Functionality and pH-dependent regulation of the Orn system are less studied, however, the presence of a system does not necessarily indicate its contribution to AR in these species. For example, putrescine is linked to the formation of biofilm (Patel et al. 2006), expression of virulence genes in *S. flexneri* (Durand and Björk 2003) and the counteraction of toxic effects caused by reactive oxygen species in *E. coli* (Jung and Kim 2003). Nevertheless, the cognate ornithine decarboxylase SpeF of the Orn system has been identified in about two-thirds (63.6%) of species harboring the ornithine/putrescine antiporter PotE, such as *E. coli*, *Shigella boydii*, *S. enterica*, *C. freundii*, and *Serratia fonticola*. Homologs of the ornithine/putrescine antiporter PotE, which belongs to the Orn system, are widely distributed and occur in species belonging to 48 different families of Proteobacteria (e.g. *Enterobacteriaceae*, *Pseudomonaceae*, *Vibrionaceae*, and *Burkholderiaceae*) and several from the phylum Firmicutes (e.g. *Streptococcaceae*, *Yersiniaceae*, *Lactobacillaceae*, and *Clostridaceae*) (see Table 2 and Fig. 5 for details). The Orn system often occurs in conjunction with the other three inducible AR systems, however, it seems to play a minor role in acid adaptation. For example, it plays only a minor role in *E. coli* MG1655 but has a more important function in avian pathogenic *E. coli*, in which growth attenuation occurs after the addition of membrane stress by sodium dodecyl sulfate. This finding suggests that PotE is more involved in membrane stress tolerance than acidic tolerance (Guerra et al. 2018). As previously mentioned, the Orn systems plays only a minor role compared to the Cad system during acid adaptation of *V. cholerae* (Merrell and Camilli 1999) (Table 2 and Fig. 5). Mutation analyses have revealed a significant role of the lysine decarboxylase *cadA*, but not the ornithine decarboxylase *speF* in both inorganic and organic acid adaptation of *V. cholerae* (Merrell and Camilli 1999). In summary, the *E. coli*-specific regulation of the Gad, Adi, Cad, and Orn systems are predominantly conserved in the *Enterobacteriaceae* family. The Gad and Orn systems occur in many bacteria, whereas the Adi and Cad systems are restricted to γ-proteobacteria. Interestingly, the decarboxylases together with their respective cognate antiporters in each system are more widely distributed than known regulatory components. The central regulators of the Cad and Adi systems identified in *E. coli* are more specific to the *Enterobacteriaceae* family.

Functional diversification of bacterial populations under acid stress

Escherichia coli possesses all four enzyme-based H^+-consuming AR systems (Gad, Adi, Cad, and Orn) and is one of the best studied organisms with respect to acid tolerance (Table 1). Individual cells do not always respond uniformly to different external pH values. The responses can be highly variable depending on the AR system, and each system is activated at different external pH values and growth phases (Fig. 3). This phenotypic heterogeneity can be beneficial for populations under acid stress. Between individual *E. coli* cells pH homeostasis varies (Martinez et al. 2012). Furthermore, persister cells of *E. coli* have a lower intracellular pH that allows survival after antibiotic treatment (Goode et al. 2021). Moreover, the induction of enzyme-based AR systems is regulated by a sophisticated interplay of membrane-integrated and soluble regulators. For example, the noisy activation of the *gadBC* promoter is highly variable under acid stress and supports the survival of single *E. coli* cells by maintaining a sufficient high intracellular pH (Mitosch et al. 2017).

Activation of the Gad, Adi, and Cad systems has been quantitatively analyzed using a fluorescent triple reporter strain under consecutively increasing acid stress conditions in *E. coli* (Brameyer et al. 2022). As shown in Fig. 6, the Cad system shows the highest heterogeneous distribution of the three systems when activated at pH 5.8 in the presence of lysine. The Adi system is activated heterogeneously under moderate acid stress (pH 4.4), though simultaneous activation of the Cad and Adi systems does not occur.

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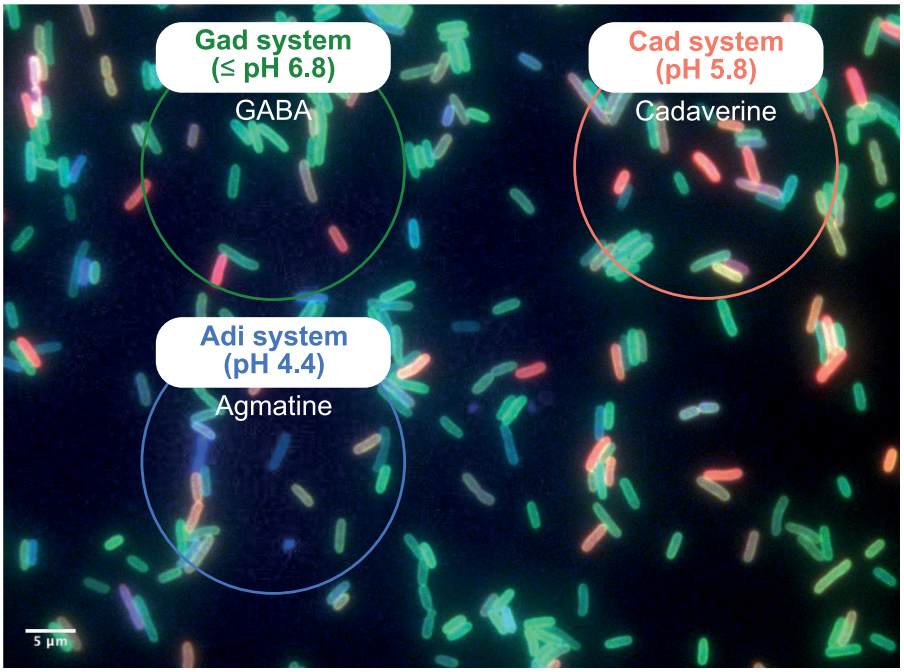

Figure 6. Model of the heterogenous activation of enzyme-based AR systems and division of labor in the *E. coli* population. Fluorescent microscopic image of the three-color reporter strain *E. coli gadC:eGFP-adiC:mCerulean-cadB:mCherry* at pH 4.4 in a complex medium. All cells of the *E. coli* population individually adapt to acid stress by activating the Gad system to varying degrees (green fluorescent cells), due to multiple extracellular and intracellular inputs, and secrete GABA. Under mild to moderate acid stress (pH 5.8–4.4), some cells in the population activate the Cad system (red fluorescent cells), while others activate the Adi system (blue fluorescent cells). These cells secrete (as indicated by the rings) the more alkaline products cadaverine and agmatine, respectively, thereby contributing to acid-stress relief with an increase of the extracellular pH, which benefits the whole population (Brameyer et al. 2022).

Under almost all conditions, bacterial cells activate the Gad system to varying degrees, primarily using intracellularly available glutamate by consuming protons to raise their internal pH. In response to high acid stress, glutamic acid is imported and GABA excreted. Heterogenous activation of the Cad and Adi systems is driven by the low copy numbers of their main regulators CadC and AdiY (Brameyer et al. 2020, 2022). Furthermore, CadC itself is distributed heterogeneously (Brameyer et al. 2019). An artificial increase in the copy number of these regulators leads to reduced heterogeneity and a growth disadvantage under acid stress, representing a metabolic burden for individual cells (Brameyer et al. 2020, 2022).

Although Adi and Cad system activation occurs only in a subpopulation of cells, the whole population benefits from the elevated external pH via secretion of the polyamine compounds cadaverine and agmatine, which are considered common goods (Fig. 6). The extent to which microcolonies of *E. coli* exist in the colon, where cadaverine and agmatine can change the micromilieu, remains unclear. Additionally, the internal pH of these cells is elevated by consuming protons during the conversion of lysine to cadaverine and arginine to agmatine, respectively (Brameyer et al. 2022). Overall, the heterogeneous activation of these three enzyme-based AR systems enables division of labor in the bacterial population, which ensures its survival over a wide range of low pH values (Fig. 6).

In contrast to *E. coli*, *V. campbellii* is more acid sensitive and uses mainly the Cad system to counteract acidic conditions. Additionally, its CadC signaling system is simpler, as CadC lacks the regulatory inputs by LysP and the polyproline motif known from *E. coli* CadC (see above). Without this tight regulation, all the cells of the *V. campbellii* population activate the Cad system, which is important, as the Cad system is the main AR system in this bacterium (Brameyer et al. 2020). Although the presence and function of the variety of AR systems are well-described in several bacterial species, their behavior at single cell level and specific niches is highly unexplored yet.

Concluding remarks

Bacteria have sophisticated and varying mechanisms for sensing and adapting to acidity. Depending on the bacterial genus, the number and complexity of AR mechanisms vary greatly, which is best reflected in the number of bacteria that survive challenging environments, such as the human stomach, and thus contribute to bacterial pathogenesis (Table 1). For example, *Escherichia* and *Shigella* spp. have the most complex regulatory network and are extremely acid-resistant, followed by *Salmonella* and *Brucella* spp., whereas *Yersinia* and *Vibrio* spp. are less acid-resistant.

At the population level, there is strong evidence for the heterogenous behavior of individual cells in response to acid stress (Mitosch et al. 2017, Smith et al. 2018, Brameyer et al. 2020, 2022). In *E. coli*, functional diversification of several AR mechanisms in single cells allows division of labor and energy conservation to ensure that at least a small percentage of the population survives even the most acidic conditions (e.g. pH 1–3). The cells' regulatory networks also affect their behavior. For example, implementing additional components or reducing the network to basic components allows heterogeneous activation of the Cad system in *Escherichia* spp., whereas it is homogeneous in *Vibrio* spp. (Brameyer et al. 2020). Further studies are required to analyze the single-cell acid stress response in more species, preferably in mixed bacterial communities and in natural environments. This analysis also could identify differences in responses to inorganic and organic acids (Wilks and Slonczewski 2007).

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pH is one of the most important parameters for bacterial interactions (Ratzke and Gore 2018). The human and animal caecum and colon are characterized by dense communities of numerous obligate anaerobic fermenting bacteria that produce a variety of short-chain fatty acids resulting in a low pH environment. Depending on the acidity and availability of amino acids (e.g. glutamic acid, lysine, arginine, and ornithine), decarboxylase-dependent systems are activated in Proteobacteria phylum members. These systems produce GABA (an important neurotransmitter); cadaverine, agmatine, and putrescine (polyamines used by prokaryotes and eukaryotes) and histamine (an allergen). However, it is unclear how these molecules change the microenvironment and to what extent they affect the host. In fact, the levels of putrescine and cadaverine are found to increase 15-fold and 3-fold, respectively, in inflamed mouse intestine monocolonized with *E. coli*, compared to an equally colonized healthy mouse cohort (Kitamoto et al. 2020). Further studies are needed to better understand the adaptation of complex communities to partially self-caused acid stress and the effects of the involved metabolites on the host.

Neutralophilic bacteria can maintain a constant intracellular pH under most conditions. However, under strong acid stress (pH < 4.4) or amplification of stress by weak organic acids, such as acetate, the cytoplasmic pH decreases (e.g. by about one pH unit in *E. coli*) (Wilks and Slonczewski 2007). A lower intracellular pH affects all macromolecules in cells and can reduce enzyme activity, acid-induced protein unfolding, membrane damage, and DNA damage. Some bacteria use low intracellular pH as a stimulus to induce other mechanisms. For example, in the acidic environment of the macrophage vacuole, *S.* Typhimurium induces virulence genes encoded in its pathogenicity island SPI-2. This induction depends on the SsrA/SsrB system, which requires acidification of the cytoplasm for activation (Liew et al. 2019). Once in the vacuole, *S.* Typhimurium acidifies its cytoplasm in an OmpR-dependent manner by repressing the CadC-regulated *cadBA* operon (Chakraborty et al. 2015). Thus, intracellular acidification of *Salmonella* spp. provides an important signal for expression of the SPI-2 type III secretion system. Recent evidence also shows that acidification of the cytoplasm in *E. coli* is linked to antibiotic resistance (Reyes-Fernández and Schuldiner 2020). The authors analyzed two mutants, derived by laboratory evolution, that are resistant to norfloxacin and chloramphenicol. They found that intracellular pH was significantly lower in the mutants than of the parental strain. The mediators of this relationship between cytoplasmic acidification and antibiotic resistance are not yet known. Interestingly, persister cells of *E. coli* display a lower intracellular pH, allowing survival after antibiotic treatment (Goode et al. 2021). Further work is needed to elucidate the mechanism by which acidification is related to virulence and antibiotic resistance.

Finally, we would like to highlight an important applied aspect for future research on acid stress resistance in bacteria. Probiotic bacteria are becoming increasingly important for their pharmaceutical value in maintaining and improving gut microbiota. So-called superfoods are marketed as products that provide nutritional value and health benefits. However, the incorporation of probiotics into food remains a challenge as the number of live bacteria declines during manufacturing and storage and especially during gastrointestinal transit (Palanivelu et al. 2022).

In summary, a better understanding of the mechanisms of AR of bacteria that have evolved during evolution has great potential for infection biology, microbiome research, and ecology.

Authors' contributions

All authors wrote the manuscript and gave final approval for publication.

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